(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 May 2016 (06.05.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/070008 A1

(51) International Patent Classification:
C12N 15/36 (2006.01) *C12Q 1/68* (2006.01)
C12N 15/62 (2006.01) *C12Q 1/66* (2006.01)
C12N 7/01 (2006.01) *C12N 15/63* (2006.01)
C12P 21/00 (2006.01) *C12N 5/10* (2006.01)
C12P 1/04 (2006.01)

(21) International Application Number:
PCT/US2015/058256

(22) International Filing Date:
30 October 2015 (30.10.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/072,679 30 October 2014 (30.10.2014) US

(71) Applicant: **THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL** [US/US]; 100 Europa Drive, Suite 430, Chapel Hill, North Carolina 27517 (US).

(72) Inventors: **SU, Lishan**; 2003 Whitmore Circle, Chapel Hill, North Carolina 27516 (US). **LI, Feng**; 2000 Baity Hill Drive, Apt. 32, Chapel Hill, North Carolina 27514 (US).

(74) Agent: **SCHWARTZMAN, Robert, A.**; Myers Bigel Sibley & Sajovec, PA, PO Box 37428, Raleigh, North Carolina 27627 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

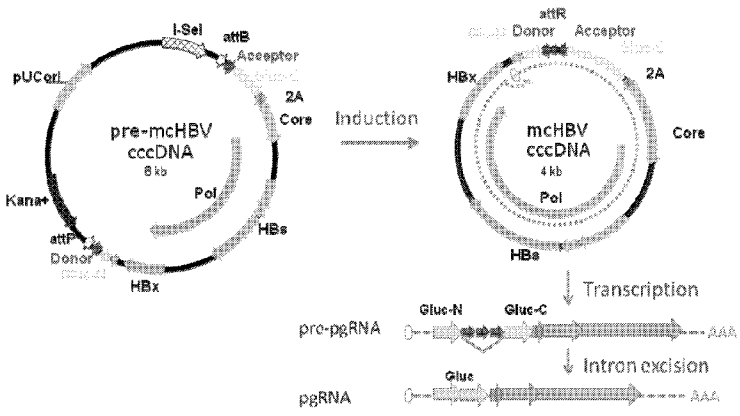
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) Title: HEPATITIS B REPORTER VIRUSES AND CCCDNA-BASED EXPRESSION VECTORS

FIG. 4A

A



(57) Abstract: The present invention relates to hepatitis B reporter viruses, systems for expression of hepatitis B virus covalently closed circular DNA, and the use thereof to produce virus, study the viral life cycle, and facilitate drug development.

WO 2016/070008 A1

Hepatitis B Reporter Viruses and cccDNA-based Expression Vectors

STATEMENT OF PRIORITY

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 62/072,679, filed October 30, 2014, the entire contents of which are incorporated by reference herein.

FIELD OF THE INVENTION

[0002] The present invention relates to hepatitis B reporter viruses, systems for expression of hepatitis B virus covalently closed circular DNA, and the use thereof to produce virus, study the viral life cycle, and facilitate drug development.

BACKGROUND OF THE INVENTION

[0003] The 3.2 kb hepatitis B virus (HBV) genome is highly compacted and economically organized. It has only 4 promoters, but generates 5 different mRNA transcripts (3.5 kb preC, 3.5 kb pregenomic, 2.4 kb, 2.1 kb, and 0.7 kb mRNA) by using different transcription start sites (Okamoto *et al., J. Virol. 68*:8102 (1994)). The genome encodes 7 functional proteins (PreCore, Core, Pol, L, M, S and X) by using ribosomal shunting (Cao *et al., J. Virol. 85*:6343 (2011)) and different translation start codons (Rehermann *et al., Nat. Rev. Immunol. 5*:215 (2005)). Much effort has been made to generate HBV vectors containing exogenous DNA in different regions of the genome including the S, preS1, preS2 and Pol genes (Chaisomchit *et al., Gene Ther. 4*:1330 (1997); Hong *et al., J. Virol. 87*:6615 (2013); Untergasser *et al., Hum. Gene Ther. 15*:203 (2004); Protzer *et al., Proc.Natl. Acad. Sci. USA 96*:10818 (1999); Klocker *et al., J. Virol. 74*:5525 (2000)). The PreS1/S2/S genes are completely embedded in the Pol gene (**Fig. 1A**), and the open reading frames (ORF) of preS1/S2/S and Pol only differ by a 1 nucleotide (nt) frameshift. In addition, the promoters of 2.4 kb and 2.1 kb mRNA reside in the Pol gene. Therefore, insertion of large DNA fragments in the preS1/S2/S and Pol overlapped region always results in translation pre-termination and/or transcription disruption, thus impairing HBV replication. Due to this complicated gene regulation, manipulating the Core gene has achieved little success (Yoo *et al., Virus Genes 24*:215 (2002); Deng *et al.,*

Hepatology 50:1380 (2009)). Recently, a very short IRES (22 nt) was engineered into the HBV sequence between the Core and Pol genes and the resulting virus was replication competent (Wang *et al., PloS one 8*:e60306 (2013)).

[0004] Current approved anti-HBV drugs include nucleotide analogs and interferon alpha (IFNa). The nucleotide analogs (*e.g.*, adefovir, entecavir, lamivudine, telbivudine, tenofovir, clevudine) work on the reverse transcription activity of HBV polymerase. The drugs can efficiently reduce HBV virus production. However, sustained virus control cannot be achieved and the virus titers rebound dramatically after withdrawing drugs. Meanwhile, the HBV can evolve and generate mutations to escape the inhibition. Long term drug use also has some side effects. The IFNa therapeutic option for HBV is inefficient in inhibiting viral production, but can achieve long lasting inhibition in responsive patients. Combination use of nucleotide analogs and IFNa achieves better outcome in those patients that respond. However, curing HBV infection is challenging using the nucleotide analogs and IFNa since they are not working directly on the HBV covalently closed circular DNA (cccDNA).

[0005] HBV cccDNA serves as a reservoir for HBV production. It exists as an episomal DNA in the nucleus of infected cells and serves as a template for HBV mRNA transcription and gene expression. The cccDNA has a very low copy number in the cells (<1 copy/cell on average). The best cure for HBV infection is to eliminate HBV cccDNA in the cells. Unfortunately, study of the biology of HBV cccDNA is still difficult for the following reasons. To specifically and reliably detect HBV cccDNA using available HBV stable cell lines is difficult due to the contamination from integrated HBV genomes or encapsidated HBV viral particles. Additionally, available HBV infection models are not conducive for screening anti-HBV cccDNA drugs due to time consuming and costly procedures.

[0006] The present invention addresses previous shortcomings in the art by providing HBV reporter viruses and systems for producing and using HBV cccDNA.

SUMMARY OF THE INVENTION

[0007] The present invention is based on the development of HBV reporter viruses, systems for producing HBV cccDNA, and the use of these products to produce virus, study the viral life cycle, and facilitate drug development.

[0008] Accordingly, in one aspect, the invention relates to a nucleic acid comprising a hepatitis B virus (HBV) genome encoding Core, PreS1/2/S, Pol, and X proteins, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the sequence encoding the Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion.

[0009] A further aspect of the invention relates to a replication competent and infectious HBV particle comprising the nucleic acid of the invention or a HBV genome produced therefrom.

[0010] Another aspect of the invention relates to a method for producing replication competent and infectious HBV particles, comprising introducing the nucleic acid of the invention into a mammalian cell under conditions suitable for HBV to replicate.

[0011] An additional aspect of the invention relates to a method for producing replication competent and infectious HBV particles, comprising introducing the nucleic acid of the invention into a mammal.

[0012] A further aspect of the invention relates to a non-human mammal model for HBV infection, comprising a non-human mammal into which the nucleic acid of the invention has been introduced.

[0013] Another aspect of the invention relates to a method for detecting protein expression from the nucleic acid of the invention or a HBV genome produced therefrom, comprising measuring activity of the reporter protein, wherein detection of reporter protein activity indicates protein expression from the nucleic acid of the invention or a HBV genome produced therefrom.

[0014] An additional aspect of the invention relates to a method for identifying a compound that modulates replication of HBV, the method comprising contacting a cell comprising the nucleic acid of the invention or a HBV genome produced therefrom with a compound, incubating the cell under conditions wherein HBV replicates in the cell in the absence of the compound; and detecting the replication level of the nucleic acid or the HBV genome produced therefrom, wherein a modulation in the replication level of the HBV genome in the cell in the presence of the compound compared to the replication level in a cell in the absence of the compound identifies the compound as a compound that modulates replication of HBV.

[0015] A further aspect of the invention relates to a pre-minicircle vector for production of a HBV cccDNA, said pre-minicircle vector comprising: a HBV genome encoding Core, PreS1/2/S, Pol, and X proteins, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the sequence encoding the HBV Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion; and an intron comprising a prokaryotic origin of replication and a marker sequence and surrounded by site-specific recombination sites inserted into the polynucleotide encoding the reporter protein.

[0016] Another aspect of the invention relates to a kit for the production of HBV cccDNA, comprising the pre-minicircle vector of the invention.

[0017] An additional aspect of the invention relates to a minicircle HBV cccDNA, said minicircle HBV cccDNA comprising: a HBV genome, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the sequence encoding the HBV Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion; wherein the minicircle HBV cccDNA is devoid of plasmid backbone DNA sequences.

[0018] A further aspect of the invention relates to a cell comprising the minicircle HBV cccDNA of the invention.

[0019] Another aspect of the invention relates to a kit for the study of HBV cccDNA, comprising the minicircle HBV cccDNA of the invention.

[0020] An additional aspect of the invention relates to a method for the production of minicircle HBV cccDNA, comprising introducing the pre-minicircle vector of the invention into bacteria to replicate the vector and culturing the bacteria under conditions suitable for site-specific recombination to occur, thereby producing minicircle HBV cccDNA.

[0021] A further aspect of the invention relates to a method for the production of detectable HBV cccDNA, comprising introducing the minicircle HBV cccDNA of the invention into a mammalian cell under conditions suitable for transcription of the minicircle HBV cccDNA.

[0022] Another aspect of the invention relates to a method for detecting protein expression from HBV cccDNA, comprising introducing the minicircle HBV cccDNA of any one of claims 39-47 into a mammalian cell under conditions suitable for

transcription of the minicircle HBV cccDNA and detecting activity and/or an amount of the reporter protein, thereby detecting expression of HBV cccDNA.

[0023] An additional aspect of the invention relates to a method for identifying a compound that modulates the amount and/or activity of HBV cccDNA, the method comprising contacting a cell comprising the minicircle HBV cccDNA of the invention with a compound, incubating the cell under conditions wherein the minicircle HBV cccDNA is expressed to produce reporter protein in the absence of the compound; and detecting activity and/or amount of the reporter protein, wherein a change in reporter protein activity and/or amount in the cell in the presence of the compound compared to the reporter protein activity and/or amount in a cell in the absence of the compound identifies the compound as a compound that modulates the level and/or activity of HBV cccDNA.

[0024] These and other aspects of the invention are set forth in more detail in the description of the invention below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **Figures 1A-1D** show generation of replication competent HBV reporter viruses. (A) Schematic representation of genome organization and gene expression of replication competent HBV. *Gaussia* luciferase (Gluc)-2A cassette is inserted in-frame into the N terminus of HBV Core gene. A segment of the first 84 nt with synonymous codons is added following the cassette to make full length Core. The pregenomic RNA (pgRNA) with the Gluc gene is generated by the CMV promoter. The organization and expression of Pol, preS1/S2/S and X genes are not altered. *, a stop codon in the core gene of the pCMV-HBV-GluMut plasmid. HBV promoters are shown as 1, 2, 3 and 4. (B, C and D). Generation of HBV viruses in NRG mice following DNA hydro-dynamically injection. (B). Representative anti-HBc staining of mouse liver tissues from different injection groups. (C) Average of Gluc activity and (D) HBV virus titers (genome equivalents/ml) in the serum at day 4 and 7 post DNA injection are shown. White bar: HBV-WT (n=7); Light Gray Bar: HBV-Gluc (n=10); Dark Gray bar: HBV-GluMut (n=8), Black bar: negative controls with no DNA injection (n=4).

[0026] **Figures 2A-2C** show characterization of recombinant HBV virions produced from stable HepG2 cell lines. Concentrated supernatants from HepG2-

HBV-WT (A), HepG2-HBV-Gluc (B), and HepG2-HBV-GlucMut (C) were subjected to iodixanol isopycnic gradient ultracentrifugation. The collected fractions were analyzed for buoyant density (g/cm^3), HBV titer (geq/ml), Gluc activity (RLU), and HBsAg (ng/ml). Mature HBV viral particles with HBV genome were negatively stained and visualized using an electron microscope (A, B). No mature viral particles were detected from cell lines expressing HBV-GlucMut (C).

[0027] **Figure 3** shows HBV-Gluc virus is infectious. Primary hepatocytes were infected with HBV-Gluc for 16h. Gluc activity was measured from triplicate wells at day 1, day 2, day 4, day 6, and day 10.

[0028] **Figures 4A-4C** show generation of minicircle HBV cccDNA (mcHBVcccDNA) and its function test. (A), Schematic representation of genome organization and gene expression. Pre-mcHBVcccDNA plasmid carries the whole HBV genome with the Gluc reporter. The Gluc was separated into Gluc-N and Gluc-C parts. Normal plasmid pre-mcHBVcccDNA can be transformed into mcHBVcccDNA by deleting the plasmid backbone part using attB and attP recombination in a special bacteria cell line. Using HBV core promoter, a pre-genomic RNA (pre-pgRNA) is generated with an intron in the middle of Gluc. Then the intron is deleted and pgRNA is formed, and Gluc-N and Gluc-C are seamlessly linked together to form a functional Gluc open reading frame. (B), DNA gel of pre-mcHBVcccDNA and mcHBVcccDNA. Pre-induced pre-mcHBVcccDNA and induced mcHBVcccDNA were analyzed using 1% agarose gel. (C), Gluc expression from mcHBVcccDNA in HepG2 cells. Equal amounts of pre-mcHBVcccDNA and mcHBVcccDNA were transfected into HepG2 cells, and the Gluc activity in the supernatant was detected and normalized to the pre-mcHBVcccDNA (as 1).

[0029] **Figure 5** shows the stability of HBV cccDNA. HepG2 cells were transfected with an equal amount of mcHBVcccDNA and mcEG (a non-HBV minicircle DNA, negative control). The Gluc activity in the supernatant was measured up to day 21. The Gluc activity was normalized to the peak level (day 3, as 1).

[0030] **Figure 6** shows the functional inhibition of HBV cccDNA by IFNa. mcHBVcccDNA was transfected into HepG2 cells. IFNa was applied 24h later at 500IU/ml and 2000IU/ml. The Gluc activity was measured at the indicated time points and normalized to before IFNa treatment (day 0).

[0031] **Figures 7A-7B** show the screening of anti HBV cccDNA ISGs (Interferon Stimulated Genes). (A), Protocol for screening. HepG2 cells were transfected with ISG and mcHBVcccDNA. Then Gluc activity was detected. rN-ZAP was used as a positive control. (B), Screening ISGs against mcHBVcccDNA. Using the protocol in (A), 288 ISG genes were screened, and Gluc activity was normalized to the pCDNA control (100%) at day 1, day 2 and day 4. Bottom line 33.3%, top line 300%.

[0032] **Figures 8A-8B** show the screening of epigenetic inhibitors against HBV cccDNA. (A), Protocol for screening epigenetic inhibitors. HepG2 cells were transfected with mcHBVcccDNA first, then epigenetic inhibitors were applied at day 3 post transfection. Gluc activity was detected at day 1 and day 2 after application. (B), A representative screening result of 73 epigenetic inhibitors. The Gluc activity was normalized to the no treatment control (as 1). Duplicated plates were used for each concentration. Top line 2 fold. Bottom line 0.3 fold.

DETAILED DESCRIPTION OF THE INVENTION

[0033] The present invention will now be described in more detail with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0034] Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a complex comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

[0035] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

[0036] Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right, unless specifically indicated otherwise. Nucleotides and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by either the one-letter code, or the three letter code, both in accordance with 37 C.F.R. §1.822 and established usage.

[0037] Except as otherwise indicated, standard methods known to those skilled in the art may be used for cloning genes, amplifying and detecting nucleic acids, and the like. Such techniques are known to those skilled in the art. *See, e.g.*, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 2nd Ed. (Cold Spring Harbor, NY, 1989); Ausubel *et al.* Current Protocols in Molecular Biology (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

[0038] All publications, patent applications, patents, patent publications and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

I. Definitions

[0039] As used in the description of the invention and the appended claims, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0040] Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

[0041] The term "about," as used herein when referring to a measurable value such as an amount of polypeptide, dose, time, temperature, enzymatic activity or other biological activity and the like, is meant to encompass variations of ± 20%, ± 10%, ± 5%, ± 1%, ± 0.5%, or even ± 0.1% of the specified amount.

[0042] The transitional phrase "consisting essentially of" means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim, "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention. *See, In re Herz*, 537 F.2d 549, 551-52, 190 USPQ 461, 463 (CCPA 1976) (emphasis in the original); *see also* MPEP § 2111.03.

[0043] The term "consists essentially of" (and grammatical variants), as applied to a polynucleotide or polypeptide sequence of this invention, means a polynucleotide or polypeptide that consists of both the recited sequence (*e.g.*, SEQ ID NO) and a total of ten or less (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) additional nucleotides or amino acids on the 5' and/or 3' or N-terminal and/or C-terminal ends of the recited sequence such that the function of the polynucleotide or polypeptide is not materially altered. The total of ten or less additional nucleotides or amino acids includes the total number of additional nucleotides or amino acids on both ends added together. The term "materially altered," as applied to polynucleotides of the invention, refers to an increase or decrease in ability to express the encoded polypeptide of at least about 50% or more as compared to the expression level of a polynucleotide consisting of the recited sequence. The term "materially altered," as applied to polypeptides of the invention, refers to an increase or decrease in at least one biological activity of at least about 50% or more as compared to the activity of a polypeptide consisting of the recited sequence.

[0044] As used herein, "nucleic acid," "nucleotide sequence," and "polynucleotide" are used interchangeably and encompass both RNA and DNA, including cDNA, genomic DNA, mRNA, synthetic (*e.g.*, chemically synthesized) DNA or RNA and chimeras of RNA and DNA. The term polynucleotide, nucleotide sequence, or nucleic acid refers to a chain of nucleotides without regard to length of the chain.

[0045] The term "isolated" can refer to a nucleic acid, nucleotide sequence or polypeptide that is substantially free of cellular material, viral material, and/or culture medium (when produced by recombinant DNA techniques), or chemical precursors or other chemicals (when chemically synthesized). Moreover, an "isolated fragment" is a fragment of a nucleic acid, nucleotide sequence or polypeptide that is not naturally occurring as a fragment and would not be found in the natural state. "Isolated" does not mean that the preparation is technically pure (homogeneous), but it is sufficiently pure to provide the polypeptide or nucleic acid in a form in which it can be used for the intended purpose.

[0046] The term "fragment," as applied to a polynucleotide, will be understood to mean a nucleotide sequence of reduced length relative to a reference nucleic acid or nucleotide sequence and comprising, consisting essentially of, and/or consisting of a

nucleotide sequence of contiguous nucleotides identical or almost identical (*e.g.*, 90%, 92%, 95%, 98%, 99% identical) to the reference nucleic acid or nucleotide sequence. Such a nucleic acid fragment according to the invention may be, where appropriate, included in a larger polynucleotide of which it is a constituent. In some embodiments, such fragments can comprise, consist essentially of, and/or consist of oligonucleotides having a length of at least about 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, or more consecutive nucleotides of a nucleic acid or nucleotide sequence according to the invention.

[0047] The term "fragment," as applied to a polypeptide, will be understood to mean an amino acid sequence of reduced length relative to a reference polypeptide or amino acid sequence and comprising, consisting essentially of, and/or consisting of an amino acid sequence of contiguous amino acids identical or almost identical (*e.g.*, 90%, 92%, 95%, 98%, 99% identical) to the reference polypeptide or amino acid sequence. Such a polypeptide fragment according to the invention may be, where appropriate, included in a larger polypeptide of which it is a constituent. In some embodiments, such fragments can comprise, consist essentially of, and/or consist of peptides having a length of at least about 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, or more consecutive amino acids of a polypeptide or amino acid sequence according to the invention.

[0048] As used herein, the terms "protein" and "polypeptide" are used interchangeably and encompass both peptides and proteins, unless indicated otherwise.

[0049] A "fusion protein" is a polypeptide produced when two heterologous nucleotide sequences or fragments thereof coding for two (or more) different polypeptides not found fused together in nature are fused together in the correct translational reading frame. Illustrative fusion polypeptides include fusions of a polypeptide of the invention (or a fragment thereof) to all or a portion of glutathione-S-transferase, maltose-binding protein, or a reporter protein (*e.g.*, Green Fluorescent Protein, β-glucuronidase, β-galactosidase, luciferase, *etc.*), hemagglutinin, c-myc, FLAG epitope, *etc.*

[0050] As used herein, a "functional" polypeptide or "functional fragment" is one that substantially retains at least one biological activity normally associated with that polypeptide (*e.g.*, target protein binding). In particular embodiments, the "functional"

polypeptide or "functional fragment" substantially retains all of the activities possessed by the unmodified peptide. By "substantially retains" biological activity, it is meant that the polypeptide retains at least about 20%, 30%, 40%, 50%, 60%, 75%, 85%, 90%, 95%, 97%, 98%, 99%, or more, of the biological activity of the native polypeptide (and can even have a higher level of activity than the native polypeptide). A "non-functional" polypeptide is one that exhibits little or essentially no detectable biological activity normally associated with the polypeptide (*e.g.*, at most, only an insignificant amount, *e.g.*, less than about 10% or even 5%). Biological activities such as protein binding can be measured using assays that are well known in the art and as described herein.

[0051] As used herein, the term "functional RNA" refers to an RNA molecule that does not encode a protein and provide a functional activity as an RNA molecule. Examples include, without limitation, RNAi, microRNA, antisense RNA, and ribozymes.

[0052] As used herein, the term "replication competent" with respect to a HBV genome refers to a genome that replicates when present in a cell (*e.g.*, a cell permissive for HBV replication). In some aspects of the present invention, replication in a cell can include the production of infectious viral particles, *i.e.*, viral particles that can infect a cell and result in the production of more infectious viral particles. The cells may specifically be cells that are maintained in culture *ex vivo*.

[0053] As used herein, the term "infectious" with respect to HBV refers to a viral particle that can infect a cell and result in the production of more infectious viral particles.

[0054] The terms "coding region" and "coding sequence" are used interchangeably and refer to a polynucleotide region that encodes a polypeptide or functional RNA and, when placed under the control of appropriate regulatory sequences, expresses the encoded polypeptide or functional RNA. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. A coding region can encode one or more polypeptides or functional RNAs. For instance, a coding region can encode a polypeptide or functional RNA that is subsequently processed into two or more polypeptides or functional RNAs. A regulatory sequence or regulatory region is a nucleotide sequence that regulates expression of a coding region to which it is

operably linked. Nonlimiting examples of regulatory sequences include promoters, transcription initiation sites, translation start sites, internal ribosome entry sites, translation stop sites, and terminators. "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory sequence is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

[0055] As used herein "sequence identity" refers to the extent to which two optimally aligned polynucleotide or polypeptide sequences are invariant throughout a window of alignment of components, *e.g.*, nucleotides or amino acids. "Identity" can be readily calculated by known methods including, but not limited to, those described in: *Computational Molecular Biology* (Lesk, A. M., ed.) Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects* (Smith, D. W., ed.) Academic Press, New York (1993); *Computer Analysis of Sequence Data, Part I* (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology* (von Heinje, G., ed.) Academic Press (1987); and *Sequence Analysis Primer* (Gribskov, M. and Devereux, J., eds.) Stockton Press, New York (1991).

[0056] As used herein, the term "substantially identical" or "corresponding to" means that two nucleic acid sequences have at least about 80% sequence identity. In some embodiments, the two nucleic acid sequences can have at least about 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity.

[0057] An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in reference sequence segment, *i.e.*, the entire reference sequence or a smaller defined part of the reference sequence. As used herein, the term "percent sequence identity" or "percent identity" refers to the percentage of identical nucleotides in a linear polynucleotide sequence of a reference ("query") polynucleotide molecule (or its complementary strand) as compared to a test ("subject") polynucleotide molecule (or its complementary strand) when the two sequences are optimally aligned (with appropriate nucleotide insertions, deletions, or gaps totaling less than 20 percent of the reference sequence over the window of comparison). In some embodiments,

"percent identity" can refer to the percentage of identical amino acids in an amino acid sequence.

[0058] Optimal alignment of sequences for aligning a comparison window are well known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman, the homology alignment algorithm of Needleman and Wunsch, the search for similarity method of Pearson and Lipman, and optionally by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the GCG® Wisconsin Package® (Accelrys Inc., Burlington, Mass.). An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in the reference sequence segment, *i.e.*, the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction multiplied by 100. The comparison of one or more polynucleotide sequences may be to a full-length polynucleotide sequence or a portion thereof, or to a longer polynucleotide sequence. For purposes of this invention "percent identity" may also be determined using BLASTX version 2.0 for translated nucleotide sequences and BLASTN version 2.0 for polynucleotide sequences.

[0059] The percent of sequence identity can be determined using the "Best Fit" or "Gap" program of the Sequence Analysis Software Package™ (Version 10; Genetics Computer Group, Inc., Madison, Wis.). "Gap" utilizes the algorithm of Needleman and Wunsch (Needleman and Wunsch, *J Mol. Biol.* 48:443-453, 1970) to find the alignment of two sequences that maximizes the number of matches and minimizes the number of gaps. "BestFit" performs an optimal alignment of the best segment of similarity between two sequences and inserts gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman (Smith and Waterman, *Adv. Appl. Math. 2*:482 (1981); Smith *et al.*, *Nucleic Acids Res. 11*:2205 (1983)).

[0060] Useful methods for determining sequence identity are also disclosed in Guide to Huge Computers (Martin J. Bishop, ed., Academic Press, San Diego (1994)), and Carillo, H., and Lipton, D., *Applied Math 48*:1073(1988)). More particularly, preferred computer programs for determining sequence identity include but are not

limited to the Basic Local Alignment Search Tool (BLAST) programs which are publicly available from National Center Biotechnology Information (NCBI) at the National Library of Medicine, National Institute of Health, Bethesda, Md. 20894; see BLAST Manual, Altschul *et al.*, NCBI, NLM, NIH; (Altschul *et al.*, *J. Mol. Biol. 215*:403 (1990)); version 2.0 or higher of BLAST programs allows the introduction of gaps (deletions and insertions) into alignments; for peptide sequence BLASTX can be used to determine sequence identity; and, for polynucleotide sequence BLASTN can be used to determine sequence identity.

II. HBV reporter viruses and methods of use

[0061] One aspect of the invention relates to a HBV genome comprising a polynucleotide encoding a reporter protein. The HBV genome produces a virus that is both replication competent and infectious. Thus, the virus is useful for studies on HBV life cycle and function as well as identification of agents that inhibit the virus and therefore may be used for treatment and/or prevention of HBV infection.

[0062] One aspect of the invention relates to a nucleic acid comprising a HBV genome encoding Core, PreS1/2/S, Pol, and X proteins, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the nucleotide sequence encoding the Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion. The HBV genome can be from any known or later identified HBV. This includes any of the known serotypes (adr, adw, ayr, ayw) and any of the known genotypes (A, B, C, D, E, F, G, or H). The HBV genome can be a wild-type genome or a genome comprising one or more naturally-occurring or non-naturally occurring mutations, *e.g.*, deletions, insertions, and/or substitutions. In some embodiments, the mutations may include point mutations, *e.g.*, deletions, insertions, and/or substitutions of a single nucleotide. In other embodiments, the mutations may include deletions, insertions, and/or substitutions of larger portions of the genome, *e.g.*, 2, 5, 10, 20, 30, 40, 50, or 100 contiguous nucleotides or more.

[0063] Polynucleotides comprising an HBV genome can be obtained from different sources, including molecularly cloned laboratory strains and clinical isolates. Clinical isolates can be from a source of infectious HBV, including tissue samples, for instance from blood, plasma, serum, liver biopsy, or leukocytes, from an infected animal, including a human or a primate. The HBV genome also can be prepared by

recombinant, enzymatic, or chemical techniques. Such methods are routine and known to the art and include, for instance, PCR.

[0064] As used herein, the term "N-terminal Core protein" refers to the portion of the Core protein that is encoded by the HBV genome that is upstream of the insertion site for the polynucleotide encoding the reporter protein. As used herein, the term "C-terminal Core protein" refers to the portion of the Core protein that is encoded by the HBV genome that is downstream of the insertion site for the polynucleotide encoding the reporter protein.

[0065] In some embodiments, the polynucleotide encoding a reporter protein is inserted in the sequence encoding the Core protein such that the reporter protein is inserted in the N-terminal portion (*e.g.*, the N-terminal half or N-terminal third) of the Core protein. In some embodiments, the polynucleotide encoding a reporter protein is inserted about 30 to about 300 nucleotides downstream of the A of the start codon of the sequence encoding the Core protein. In certain embodiments, the polynucleotide encoding a reporter protein is inserted about 50 to about 200, *e.g.*, 75 to about 100, *e.g.*, about 80 to about 90, nucleotides downstream of the A of the start codon of the sequence encoding the Core protein, *e.g.*, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 nucleotides downstream of the A of the start codon of the sequence encoding the Core protein, *e.g.*, 84 nucleotides downstream of the A of the start codon of the sequence encoding the Core protein.

[0066] In some embodiments, the HBV genome is further modified to encode a Core fusion protein wherein the N-terminal Core protein located on the N-terminal side of the reporter protein is duplicated on the C-terminal side of the reporter protein. In other words, the fusion protein comprises a full-length Core protein on the C-terminal side of the reporter protein. The duplicated N-terminal Core protein is referred to herein as N-coreOpt to distinguish it from the N-terminal Core protein located on the N-terminal side of the reporter protein. In certain embodiments, this modification is carried out by inserting a polynucleotide encoding the N-coreOpt protein (*e.g.*, a synthetic, *i.e.*, artificial or non-naturally occurring, polynucleotide) in-frame between the polynucleotide encoding the reporter protein and the sequence encoding the C-terminal Core protein. In order to prevent undesired recombination between the two polynucleotides encoding the N-terminal Core protein and the N-coreOpt, the inserted polynucleotide encoding the N-coreOpt can differ in nucleotide

sequence from the polynucleotide sequence encoding the N-terminal Core protein while encoding the same amino acid sequence, *i.e.*, using synonymous codons. In certain embodiments, the polynucleotide encoding the N-coreOpt is no more than about 95% identical to the polynucleotide encoding the N-terminal core protein, *e.g.*, no more that about 90%, 85%, 80%, 75%, or 70% identical.

[0067] The reporter protein can be any reporter protein that can be specifically detected when expressed. In some embodiments, the reporter protein is one that is secreted from an HBV-infected cell. Suitable reporter proteins include, without limitation, a fluorescent protein (*e.g.*, EGFP, GFP, RFP, BFP, YFP, or dsRED2), an enzyme that produces a detectable product, such as luciferase (*e.g.*, from *Gaussia*, *Renilla*, or *Photinus*), β-galactosidase, β-glucuronidase, alkaline phosphatase, and chloramphenicol acetyltransferase gene, or proteins that can be directly detected. Virtually any protein can be directly detected by using, for example, specific antibodies to the protein. Additional markers (and associated antibiotics) that are suitable for either positive or negative selection of eukaryotic cells are disclosed in Sambrook and Russell (2001), *Molecular Cloning*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Ausubel et al. (1992), *Current Protocols in Molecular Biology*, John Wiley & Sons, including periodic updates. Any of the disclosed markers, as well as others known in the art, may be used to practice the present invention.

[0068] In certain embodiments, the polynucleotide encoding the reporter protein is no longer than about 1,000 nucleotides, *e.g.*, no longer than about 900, 800, 700, 600, or 500 nucleotides.

[0069] In some embodiments, the HBV genome is operably linked to a promoter. Any promoter suitable for expression of the HBV genome may be used, *e.g.*, a cytomegalovirus promoter.

[0070] The nucleic acid comprising a HBV genome can be in the form of any vector suitable for expression of the HBV genome. A vector is a replicating polynucleotide, such as a plasmid, phage, cosmid, or artificial chromosome to which another polynucleotide may be attached so as to bring about the replication of the attached polynucleotide. A vector can provide for further cloning (amplification of the polynucleotide), *i.e.*, a cloning vector, or for expression of the polypeptide or functional RNA encoded by the coding region, *i.e.*, an expression vector. In some

embodiments, the vector is a plasmid. In some embodiments, the vector is able to replicate in a prokaryotic host cell, for instance *Escherichia coli*, and/or able to replicate in a eukaryotic cell. In certain embodiments, the vector can integrate in the genomic DNA of a eukaryotic cell.

[0071] Another aspect of the invention is a replication competent and infectious HBV particle comprising the nucleic acid of the invention or a HBV genome produced therefrom.

[0072] Another aspect of the invention relates to a non-human animal model for HBV infection, in which the animal disease model comprises a hepatitis B virus genome in the liver cells thereof and exhibits one or more of the following symptoms: a) HBV viral particles in the serum; b) HBV viral DNA in the serum; c) HBV envelope and HBV e proteins in the serum; and d) expression of HBV core and HBV envelope proteins in the liver. In certain embodiments, the non-human animal is a primate, a dog, or a rodent, *e.g.*, a mouse. The non-human animal can be immunocompetent (e.g., BALB/c, ICR, C57BL/6 and FVB mouse strains) or immunodeficient (*e.g.*, NRG mouse strain).

[0073] A further aspect of the invention relates to a method of generating a non-human animal disease model for HBV infection, comprising obtaining a non-human animal and administering to the animal a modified HBV genome of the invention. In certain embodiments, the modified HBV genome is administered intravenously, *e.g.*, using hydrodynamic injection.

[0074] Further aspects of the invention relate to methods of using the modified HBV genomes of the invention to produce viral particles, to study aspects of the viral life cycle, for drug development, to study viral resistance to therapeutics, and/or for any other suitable use.

[0075] One aspect of the invention relates to a method for replicating a replication competent and infectious HBV reporter virus, comprising incubating a cell comprising the modified HBV genome of the invention under conditions suitable for the genome to replicate. Any suitable method for delivering the modified HBV genome to the cell may be used as is well known in the art and described herein. Such methods include, for instance, liposome and non-liposome mediated transfection. Non-liposome mediated transfection methods include, for instance,

electroporation. Conditions suitable for HBV replication are well known in the art and described herein.

[0076] An additional aspect of the invention relates to a method for producing viral particles, comprising incubating a cell comprising the modified HBV genome of the invention under conditions suitable for the genome to replicate. In some embodiments, the method further comprises isolating the viral particles from the cell. Conditions suitable for HBV replication and virus production are well known in the art and described herein. Any suitable cell can be used, *e.g.*, a hepatic cell, *e.g.*, a primary human hepatocyte, human hepatoma cell (such as Huh-7.5, Huh-7, HepRG, HepG2, IMY-N9, PH5CH8), or other hepatocyte-derived cell, *e.g.*, a hepatocyte-derived cell line.

[0077] The viral particles can be used as a source of virus particles for various assays, including evaluating methods for inactivating particles, excluding particles from serum, identifying a neutralizing compound, and as an antigen for use in detecting anti-HBV antibodies in an animal. An example of using a viral particle as an antigen includes use as a positive-control in assays that test for the presence of anti-HBV antibodies.

[0078] For example, the activity of compounds that neutralize or inactivate the particles can be evaluated by measuring the ability of the molecule to prevent the particles from infecting cells growing in culture or in cells in an animal. Inactivating compounds include detergents and solvents that solubilize the envelope of a viral particle. Inactivating compounds are often used in the production of blood products and cell-free blood products. Examples of compounds that can be neutralizing include a polyketide, a non-ribosomal peptide, a polypeptide (for instance, an antibody), a polynucleotide (for instance, an antisense oligonucleotide or ribozyme), or other organic molecules.

[0079] In some embodiments, the modified HBV genome of the invention may be used in screening assays to identify agents that modulate HBV replication and viral particle production.

[0080] In one embodiment, the invention relates to a method for detecting protein expression from the nucleic acid of the invention or a HBV genome produced therefrom, comprising detecting activity and/or an amount of the reporter protein, wherein detection of reporter protein activity and/or amount indicates protein

expression from the nucleic acid of the invention or a HBV genome produced therefrom. As used herein, the term "protein expression from the nucleic acid" means that the protein is produced from the nucleic acid encoding the protein.

[0081] In one embodiment, the invention relates to a method for identifying a compound that modulates replication of HBV, the method comprising contacting a cell comprising the nucleic acid of the invention or a HBV genome produced therefrom with a compound, incubating the cell under conditions wherein HBV replicates in the cell in the absence of the compound; and detecting the replication level of the nucleic acid or the HBV genome produced therefrom, wherein a modulation in the replication level of the HBV genome in the cell in the presence of the compound compared to the replication level in a cell in the absence of the compound identifies the compound as a compound that modulates replication of HBV.

[0082] In another embodiment, the invention relates to a method for identifying a compound that modulates replication of HBV, the method comprising administering to a non-human animal comprising the nucleic acid of the invention or a HBV genome produced therefrom a compound; and detecting the replication level of the nucleic acid or a HBV genome produced therefrom, wherein a modulation in the replication level of the HBV genome in the non-human animal administered the compound compared to the replication level in a non-human animal not administered the compound indicates the compound modulates replication of HBV.

[0083] A compound that modulates replication of HBV includes compounds that completely prevent replication, as well as compounds that decrease replication or increase replication. In certain embodiments, a compound modulates replication of HBV by at least about 50%, *e.g.*, at least about 75%, *e.g.*, at least about 95%.

[0084] The compounds added to a cell can be a wide range of molecules and is not a limiting aspect of the invention. Compounds include, for instance, a polyketide, a non-ribosomal peptide, a polypeptide, a polynucleotide (for instance an siRNA, antisense oligonucleotide or ribozyme), other organic molecules, or a combination thereof. The sources for compounds to be screened can include, for example, chemical compound libraries, fermentation media of Streptomycetes, other bacteria and fungi, and extracts of eukaryotic or prokaryotic cells. When the compound is added to the cell is also not a limiting aspect of the invention. For instance, the

compound can be added to a cell that contains a modified HBV genome. Alternatively, the compound can be added to a cell before or at the same time that the modified HBV genome is introduced to the cell.

[0085] Detection of the replication level of the genome may be carried out by any method known in the art or described herein. In certain embodiments, detecting the replication level of the modified HBV genome comprises detecting the activity and/or quantity of the reporter protein or, alternatively, detecting the quantity of viral RNA (*e.g.*, using nucleic acid amplification assays such as PCR), detecting the quantity of viral protein (*e.g.*, using immunoassays), detecting the quantity of a marker protein, or detecting the quantity of infectious virus particles (*e.g.*, using an infectivity assay).

[0086] In each of these methods, any suitable cell can be used, *e.g.*, primate or human cells, *e.g.*, a hepatic cell, *e.g.*, a primary human hepatocyte, human hepatoma cell (such as Huh-7.5, Huh-7, HepRG, HepG2, IMY-N9, PH5CH8), or other hepatocyte-derived cell line.

[0087] A further aspect of the invention relates to a kit comprising the HBV reporter virus of the invention. The kit may further comprise additional components for use of the HBV reporter virus of the invention, *e.g.*, buffers, reagents, enzymes, substrates, containers, bacteria, cells, instructions, *etc.*

III. HBV cccDNA minicircles and methods of use

[0088] One aspect of the invention relates to the development of techniques for producing HBV cccDNA using minicircles. The availability of large amounts of cccDNA permits the study of the HBV lifecycle and the screening of drugs for treatment and/or prevention of HBV infection (*e.g.*, prevention of a disease or disorder caused by HBV infection).

[0089] The HBV cccDNA system is based on the HBV reporter virus described above. Starting with the reporter virus, an intron is inserted into the polynucleotide encoding the reporter protein. The split HBV reporter virus is incorporated into a minicircle vector to generate a pre-minicircle HBV cccDNA. Removal of the intron generates a minicircle HBV cDNA comprising a short DNA sequence, herein referred to as a splice residue, in the polynucleotide encoding the reporter protein which is the remaining nucleotides from the intron and recombination sites following splicing. A unique feature of the cccDNA system is that the minicircle produces a pre-

pregenomic RNA using the HBV promoter. The splice residue DNA sequence from the intron and recombination sites is spliced out during maturation of the pre-pregenomic RNA into the pregenomic RNA, in which the polynucleotide encoding the reporter protein is brought together for production of the reporter protein. This cccDNA system provides efficient production of cccDNA accompanied by a detectable reporter for monitoring cccDNA activity.

[0090] Thus, one aspect of the invention relates to a pre-minicircle vector for production of a HBV cccDNA, said pre-minicircle vector comprising:

a HBV genome encoding Core, PreS1/2/S, Pol, and X proteins, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the sequence encoding the HBV Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion; and

an intron comprising a prokaryotic origin of replication and a marker sequence and surrounded by site-specific recombination sites inserted into the polynucleotide encoding the reporter protein. The term "surrounded" as used herein refers to the presence of site-specific recombination sites on the 5' and 3' ends of the intron.

[0091] The HBV genome can be from any known or later identified HBV. This includes any of the known serotypes (adr, adw, ayr, ayw) and any of the known genotypes (A, B, C, D, E, F, G, or H). The HBV genome can be a wild-type genome or a genome comprising one or more naturally-occurring or non-naturally occurring mutations, *e.g.*, deletions, insertions, and/or substitutions. In some embodiments, the mutations may include point mutations, *e.g.*, deletions, insertions, and/or substitutions of a single nucleotide. In other embodiments, the mutations may include deletions, insertions, and/or substitutions of larger portions of the genome, *e.g.*, 2, 5, 10, 20, 30, 40, 50, or 100 contiguous nucleotides or more.

[0092] The minicircle vector may be any type of vector known in the art. In some embodiments, the vector is a plasmid. Minicircles vectors can be constructed as known in the art and described herein (*see, e.g.*, Darquet *et al.*, *Gene Ther.* 4:1341 (1997); Darquet *et al.*, *Gene Ther.* 6:209 (1999); Bigger *et al.*, *J. Biol. Chem.* 276:23018 (2001)).

[0093] The marker sequence can be any marker sequence recognized in the art. In some embodiments, the marker sequence is an antibiotic resistance gene, heavy metal

resistance gene, auxotrophic selection marker, or a gene which produces a substance necessary for bacterial growth.

[0094] The prokaryotic origin of replication can be any prokaryotic origin of replication recognized in the art. In certain embodiments, the prokaryotic origin of replication is a high copy number origin of replication, *e.g.*, one from plasmid pUC19.

[0095] Site-specific recombination sites are well known in the art. Examples include, without limitation, attB, attP, loxP sites, γδ res sites, FRT sites, hixL, hixR, TN3 res sites, Tn21 res sites, psi sites, and cer sites. These sites can be used in any combination suitable for removal of the intron.

[0096] In certain embodiments, the intron comprises additional functional sequences, such as a polynucleotide encoding a site-specific recombinase, *e.g.*, one that recognizes the site-specific recombination sites surrounding the intron. Examples of recombinases suitable for the invention include, without limitation, γδ resolvase, Hin recombinase, P1 Cre, yeast 2 micron Flp, Tn3 resolvase, Tn21 resolvase, λ integrase, and XerCD.

[0097] In other embodiments, the bacteria used for generating the minicircle express the site-specific recombinase, *e.g.*, the bacteria are genetically modified to express the site-specific recombinase.

[0098] In some embodiments, the intron further comprises an inducible lysis gene to promote breaking open (lysing) the bacteria after production of the minicircle. An example of an inducible lysis gene is lysis gene E of bacteriophage PhiX174.

[0099] Another aspect of the invention relates to a minicircle HBV cccDNA, said minicircle HBV cccDNA comprising:

a HBV genome, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the sequence encoding the HBV Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion;

wherein the minicircle HBV cccDNA is devoid of plasmid backbone DNA sequences.

[0100] The term "devoid of plasmid backbone DNA sequences" as used herein refers to a minicircle HBV cccDNA that contains less than 5% of plasmid sequences, including the intron and surrounding site-specific recombination sites, *e.g.*, less than 4%, 3%, 2%, or 1%.

[0101] In some embodiments, the minicircle HBV cccDNA further comprises a splice residue resulting from site-specific recombination inserted into the polynucleotide encoding the reporter protein. The splice residue is the short DNA sequence that is the residue from the intron and recombination sites after the intron has been removed by recombination. For example, when the site-specific recombination sites are attP and attB, the product hybrid sequence comprises the residue attR or attL as well as the donor and acceptor sites. The splice residue is removed during maturation of the pre-pregenomic RNA produced form the minicircle into the pregenomic RNA

[0102] A further aspect of the invention relates to a cell comprising the minicircle cccDNA of the invention. In some embodiments, the cell is a cell capable of supporting transcription of the cccDNA. In certain embodiments, the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell.

[0103] An additional aspect of the invention relates to a method for the production of minicircle HBV cccDNA, comprising introducing the pre-minicircle vector of the invention into bacteria to replicate the vector and culturing the bacteria under conditions suitable for site-specific recombination to occur, thereby producing minicircle HBV cccDNA. In some embodiments, the bacteria express a site-specific recombinase. In other embodiments, the pre-minicircle vector encodes a site-specific recombinase that is expressed upon introduction of the pre-minicircle vector into the bacteria. The method may further comprise a step of isolating the minicircle HBV cccDNA produced in the bacteria. The minicircle may be isolated by methods known in the art for separation of vectors, such as a chromatography process using a column comprising an ion exchange matrix, a matrix for hydrophobic interaction chromatography, or a matrix for size exclusion chromatography. In one embodiment, the minicircle further comprises an identification sequence (*e.g.*, a lac operator site) that can be used to isolate the minicircle, *e.g.*, by affinity chromatography.

[0104] Another aspect of the invention relates to a method for the production of detectable HBV cccDNA, comprising introducing the minicircle HBV cccDNA of the invention into a mammalian cell under conditions suitable for transcription of the minicircle HBV cccDNA. In some embodiments, the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell. In other

embodiments, the cell is present in a mammal, *e.g.*, a human or a non-human mammal, *e.g.*, an animal model of hepatitis infection.

[0105] In the methods of the invention, the vector is introduced into a target cell. Any convenient protocol may be employed, where the protocol may provide for *in vitro* or *in vivo* introduction of the vector into the target cell, depending on the location of the target cell. For example, where the target cell is an isolated cell, the vector may be introduced directly into the cell under cell culture conditions permissive of viability of the target cell, *e.g.*, by using standard transformation techniques. Such techniques include, but are not limited to, viral infection, transformation, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate precipitation, direct microinjection, viral vector delivery, use of nanoparticles, and the like. The choice of method is generally dependent on the type of cell being transformed and the circumstances under which the transformation is taking place (*i.e.*, *in vitro*, *ex vivo*, or *in vivo*). A general discussion of these methods can be found in Ausubel et al., *Short Protocols in Molecular Biology*, 3rd ed., Wiley & Sons, 1995.

[0106] Alternatively, where the target cell or cells are part of a multicellular organism, the vector may be administered to the organism or host in a manner such that the vector is able to enter the target cell(s), *e.g.*, via an *in vivo* or *ex vivo* protocol. By "*in vivo*," it is meant that the vector is administered to a living body of an animal. By "*ex vivo*," it is meant that cells or organs are modified outside of the body. Such cells or organs are typically returned to a living body. Methods for the administration of nucleic acid constructs are well known in the art and include use of nanoparticles as described in Bharali *et al.*, *Proc. Natl. Acad. Sci. USA* 102(32):11539-44 (2005). Nucleic acid constructs can be delivered with cationic lipids (Goddard *et al.*, *Gene Ther.* 4:1231-1236 (1997); Gorman *et al.*, *Gene Ther.* 4:983-992 (1997); Chadwick *et al.*, *Gene Ther.* 4:937-942 (1997); Gokhale *et al.*, *Gene Ther.* 4:1289-1299 (1997); Gao *et al.*, *Gene Ther.* 2:710-722 (1995)), using viral vectors (Monahan *et al.*, *Gene Ther.* 4:40-49 (1997); Onodera *et al.*, *Blood* 91:30-36 (1998)), by uptake of "naked DNA", and the like. Techniques well known in the art for the transformation of cells can be used for the *ex vivo* administration of nucleic acid constructs. The exact formulation, route of administration and dosage can be chosen empirically. (See, *e.g.*, Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics," Ch. 1 pl).

[0107] The route of administration of the vector to the multicellular organism depends on several parameters, including: the nature of the vectors that carry the system components, the nature of the delivery vehicle, the nature of the multicellular organism, and the like, where a common feature of the mode of administration is that it provides for *in vivo* delivery of the vector components to the target cell(s) via a systemic route. Of particular interest as systemic routes are vascular routes, by which the vector is introduced into the vascular system of the host, *e.g.*, an artery or vein, where intravenous routes of administration are of particular interest in many embodiments.

[0108] The particular dosage of vector that is administered to the multicellular organism in the subject methods varies depending on the nature of vector, the nature of the expression module and gene, the nature of the delivery vehicle and the like. Dosages can readily be determined empirically by those of skill in the art. For example, in mice where the vectors are intravenously administered in a saline solution vehicle, the amount of vector that is administered in many embodiments typically ranges from about 2 to about 100 and usually from about 10 to about 50 μg.

[0109] A further aspect of the invention relates to a method for detecting protein expression from HBV cccDNA, comprising introducing the minicircle HBV cccDNA of the invention into a mammalian cell under conditions suitable for transcription of the minicircle HBV cccDNA and measuring activity and/or amount of the reporter protein. In some embodiments, the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell. Measuring the activity and/or amount of the reporter protein may comprise measuring the activity and/or amount in culture supernatant if the reporter protein is secreted form the cell. In other embodiments, the cell is present in a mammal, *e.g.*, a human or a non-human mammal, *e.g.*, an animal model of hepatitis infection. In these embodiments, measuring the activity and/or amount of the reporter protein may comprise measuring the activity and/or amount in a sample from the mammal. The sample may be, without limitation, blood, serum, plasma, saliva, urine, tissue samples or biopsy material (*e.g.*, from liver).

[0110] The HBV cccDNA of the invention is advantageously used in studies of the HBV lifecycle and the screening of drugs for treatment and/or prevention of HBV infection. Thus, one aspect of the invention relates to a method for identifying a

compound that modulates the amount and/or activity of HBV cccDNA, the method comprising contacting a cell comprising the minicircle HBV cccDNA of the invention with a compound, incubating the cell under conditions wherein the minicircle HBV cccDNA is expressed to produce reporter protein in the absence of the compound; and detecting activity and/or amount of the reporter protein, wherein a change in reporter protein activity and/or amount in the cell in the presence of the compound compared to the reporter protein activity and/or amount in a cell in the absence of the compound identifies the compound as a compound that modulates the level and/or activity of HBV cccDNA. In some embodiments, the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell. Measuring the activity and/or amount of the reporter protein may comprise measuring the activity and/or amount in culture supernatant if the reporter protein is secreted form the cell. In other embodiments, the cell is present in a mammal, *e.g.*, a human or a non-human mammal, *e.g.*, an animal model of hepatitis infection. In these embodiments, measuring the activity and/or amount of the reporter protein may comprise measuring the activity and/or amount in a sample from the mammal. The sample may be, without limitation, blood, serum, plasma, saliva, urine, tissue samples or biopsies (*e.g.*, from liver).

[0111] A further aspect of the invention relates to a kit comprising the pre-minicircle vector and/or minicircle HBV cccDNA of the invention. The kit may further comprise additional components for use of the pre-minicircle vector and/or minicircle HBV cccDNA of the invention, *e.g.*, buffers, reagents, enzymes, substrates, containers, bacteria, cells, instructions, *etc.*

[0112] The present invention is more particularly described in the following examples that are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLE 1

HBV Reporter Virus

[0113] A replication competent HBV was generated by inserting foreign DNA fragments at the N-terminus of the Core gene (**Fig. 1A**). The pCMV-HBV-WT plasmid contains a full-length HBV genome which was generated from HBV genotype C2 plasma using a modified overlap PCR method (Hu *et al., J. Virol. Meth.*

161:63 (2009)). Then the *Gaussia* luciferase (Gluc) gene was inserted to generate pCMV-HBV-Gluc. Considering the Core and Pol are coordinately and precisely translated from the same pgRNA using a ribosome shunting strategy (Cao *et al., J. Virol. 85*:6343 (2011)) and the failure to generate useful HBV vector by simple replacement of the whole Core gene with EGFP (Yoo *et al., Virus Genes 24*:215 (2002)), Gluc-2A was put behind the +84 nt site (the A of the initial ATG of Core as +1) to maximally maintain the native mRNA structure required for ribosome shunting for Pol translation (Li *et al., J. Virol. 84*:12075 (2010)). To maintain the integrity of Core protein, a synthesized 84 nt DNA with synonymous codons expressing the first 18 amino acids (a.a.) of the Core was put behind Gluc-2A. Therefore, the potential genome instability due to otherwise repeat sequences (84 nt) flanking Gluc-2A insert is avoided by the synonymous codons. The Core (18 a.a.)-Gluc-2A-Core(full length) is expressed from the pgRNA as a fusion protein using the native ATG of Core, and the 2A separates the full-length Core protein from the Core(18 a.a.)-Gluc-2A. This design will maintain the rest of the gene expression the same as the wild type HBV without altering the HBV promoters for the 2.4kb, 2.1kb, and 0.9kb mRNAs (**Fig. 1A**). Meanwhile, a Core deficient plasmid pCMV-HBV-GlucMut was constructed by inserting a stop codon before the Core gene (*, **Fig. 1A**), thus no virus will be produced.

[0114] To test whether HBV-Gluc virus can be produced, the pCMV-HBV-WT, pCMV-HBV-Gluc and pCMV-HBV-GlucMut were hydrodynamically injected into immune deficient NOD-*Rag1-/-IL2RgammaC-null* (NRG) mice, through tail vein at a dose of 10 μg plasmid/mouse (Chang *et al., J. Hepatol. 39*:834 (2003)). pCMV-HBV-WT and pCMV-HBV-Gluc, but not pCMV-HBV-GlucMut, produced the Core protein in mouse liver as examined by immunostaining with anti-HBc antibody (1:100, Zeta Corp) (**Fig. 1B**). No difference in the Gluc expression (Promega kit, E1960) was observed between the mice injected with pCMV-HBV-Gluc and pCMV-HBV-GlucMut, and the Gluc expression decreased from day 4 to day 7 (**Fig. 1C**). Meanwhile, the virus titers with HBV genome in mice injected with HBV-WT and HBV-Gluc increased from day 4 to day 7 (**Fig. 1D**), indicating that the HBV-Gluc mice are producing genome-containing HBV virions.

[0115] To generate HBV-Gluc virus in HepG2 cells, pCMV-HBV-WT, pCMV-HBV-Gluc and pCMV-HBV-GlucMut were linearized and transfected (Amaxa

Nucleotransfection Kit V, program H-022, Lonza) into HepG2 cells to establish stable cell lines HepG2-HBV-WT, HepG2-HBV-Gluc and HepG2-HBV-GlucMut. For each plasmid, multiple clones were obtained and their viral yields were assessed. Since the viral yields among different cell clones were almost identical, only one cell clone from each plasmid was selected for further characterization. Cell culture supernatants were concentrated using 6% PEG8000, followed by iodixanol isopycnic gradient ultracentrifugation (Beckman SW41 rotor 34000rpm, 24h) (Feng *et al., Nature 496*:367 (2013)). 0.5 ml fractions were collected. The buoyant density was determined using a refractometer (Fig. 2) (Feng *et al., Nature 496*:367 (2013)). The HBsAg, HBV titers and Gluc activity were measured (**Fig. 2**). The density of HBV-Gluc viral particles is the same as that of wild type HBV in fraction 13 (1.177 versus 1.171 g/cm^3) (**Figs. 2A and 2B**). No viral particles were detected in the HepG2-HBV-GlucMut (**Fig. 2C**). All three cell lines produced subviral particles and a similar level of Gluc was expressed from HBV-Gluc and HBV-GlucMut cell lines. Fraction 13 containing HBV genome was further examined by electron microscope (EM) using negative staining (Feng *et al., Nature 496*:367 (2013)). Mature HBV particles (42 nm) were observed both from HBV-WT and HBV-Gluc viruses, but no mature virus particles were detected from HBV-GlucMut.

[0116] The virus yield of HBV-Gluc was significantly lower than that of the wild type HBV (**Fig. 1D**), which, we thought, was attributed to the long insert size (**Table 1**). However, the relationship of HBV virus yield and insert size has not been thoroughly investigated (Chaisomchit *et al., Gene Ther. 4*:1330 (1997); Hong *et al., J. Virol. 87*:6615 (2013); Untergasser *et al., Hum. Gene Ther. 15*:203 (2004); Protzer *et al., Proc.Natl. Acad. Sci. USA 96*:10818 (1999); Klocker *et al., J. Virol. 74*:5525 (2000); Yoo *et al., Virus Genes 24*:215 (2002); Deng *et al., Hepatology 50*:1380 (2009); Wang *et al., PloS one 8*:e60306 (2013)). To test whether the length of the insert affects the replication efficiency, we replaced the Gluc (717 bp) sequence with a mouse HSA gene insert (393 bp). The virus yields from mouse serum, plasmid transfection (huh7.5), and HepG2 stable cell lines were compared (**Table 1**). A clear size-dependent virus yield was shown.

Table 1. Summary of the size effect of exotic DNA insertion on HBV yields in different systems.

	HBV-WT	HBV-HSA	HBV-Gluc
Insert size (bp)[a]	0	393	717
Virus yields[b]			
Hydrodynamic injection			
Mouse number	7	7	10
Day4			
avg ± SD (geq/ml)	7.53 ± 8.32e6	7.68 ± 6.01e5	3.37 ± 3.55e4
Relative yields (%)	100	10.2	0.4
Plasmid transfection[c] (Huh7.5)			
Day4			
geq/ml	8.66 e7	1.32e7	3.50e6
Relative yields (%)	100	15.2	4.0
HepG2 stable cell line[c,d]			
Day5			
avg ± SD (geq/ml)	2.07 ± 0.62e7	4.46 ± 1.11 e6	5.20 ± 0.67 e5
Relative yields (%)	100	21.5	2.5

[a] All the exogenous DNA includes HSA or Gluc, 2A and 84nt Core sequence with the synonymous mutated codons.

[b] Virus yields are calculated using quantitative PCR, gep/ml represents the genomic equivalents/ml. Relative yields are normalized to wild type HBV as 100%.

[c] Virus titer are calculated by dividing the sum of virus containing Fraction 12,13,and 14 in Figure 2 by the initial total volume of culture supernatant.

[d] Average of two independent experiment.

[0117] The infectivity of the HBV-Gluc was tested using primary hepatocytes. Primary hepatocytes were infected with HBV-Gluc for 16h. The infection inoculums

were extensively washed. An increased level of Gluc in the supernatant was observed (**Fig. 3**), indicating the HBV-Gluc virus is infectious.

EXAMPLE 2

Generation of HBV cccDNA expression system

[0118] In order to study the HBV cccDNA, a HBV cccDNA system was generated. Based on the HBV-Gluc reporter virus of Example 1, an intron was inserted into HBV Gluc. The split HBV reporter virus was ligated into a minicircle plasmid to get a pre-mcHBVcccDNA as shown (**Fig. 4A**). Gluc cannot be expressed due to its split into Gluc-N and Gluc-C parts. The mcHBVcccDNA can be produced through attB and attP recombination in a special bacteria cell line. Most of the plasmid backbone is circled away and digested. Only the mcHBVcccDNA was left. Only a short DNA sequence including an intron and residue attR was kept in the mcHBVcccDNA. The special part of this design is that a pre-pregenomic RNA can be generated using the HBV promoter. The intron splicing donor-attR-acceptor sequence will be spliced off during the pre-pgRNA maturation into pgRNA, in which the Gluc-N and Gluc-C are finally linked together without any extra sequence. Therefore Gluc can be produced. As Gluc can be secreted, Gluc activity in the culture supernatant can indicate the activity of mcHBVcccDNA. mcHBVcccDNA can be efficiently produced (**Fig. 4B**). Additionally, the Gluc activity can be detected when mcHBVcccDNA is transfected into HepG2 cells (**Fig. 4C**).

[0119] In the HBV patient, nucleotide analogues are very effective to prevent production of new HBV virus. However, the HBV will rebound quickly after inhibitor withdrawal. Even the combination of nucleotide analogues and IFNa cannot efficiently reduce cccDNA level. Therefore, HBV cccDNA is supposed to be very stable *in vivo*. In an infection model (using HepaRG or HepG2-NTCP cell lines), cccDNA can be maintained for a long time. In the next experiment, mcHBVcccDNA was transfected into HepG2 cells, and the Gluc activity was measured for up to 3 weeks. Meanwhile, a non-HBV minicircle DNA, mcEG containing Gluc and EGFP, was used as a negative control. The Gluc activity was normalized to the peak level (day 3, set as 1). In the mcEG control group, the Gluc activity decreased quickly from 1 (day 3) to less than 0.1 (day 7) in 4 days (**Fig. 5**). However, with mcHBVcccDNA the Gluc activity was maintained to day 5, and slow decreased to 0.5

fold until day 21 (**Fig. 5**). The data indicated that mcHBVcccDNA can be used to study the function of HBV cccDNA.

[0120] IFNa can inhibit the activity of cccDNA in responsive HBV patients. In the next experiment IFNa was applied to HepG2 transfected with mcHBVcccDNA at a dose of 500IU/ml and 2000IU/ml. HepG2 cells were transfected with mcHBVcccDNA first, and IFNa was added at 24h post transfection. A dose dependent inhibition of Gluc activity was observed after 84 h treatment, suggesting that mcHBVcccDNA can serve as a good surrogate for studying real HBV cccDNA (**Fig. 6**).

EXAMPLE 3

Screening assays using HBV cccDNA expression system

[0121] Secreted Gluc can be easily detected and quantitated and the culture supernatant can be taken repeatedly from the same well over time, which makes the cccDNA expression system an ideal reporter for high throughput drug screening. A pilot study was performed to test the feasibility. An ISG (interferon stimulated genes) library containing 288 genes was chosen. In the pilot study, HepG2 cells were transfected with rN-ZAP gene and mcHBVcccDNA (**Fig. 7A**). rN-ZAP has been demonstrated to be able to degrade HBV mRNA. To guarantee every mcHBVcccDNA transfected cell contained ISG genes, a ratio of 8:1 (ISG vs. mcHBVcccDNA) was used. It was shown that Gluc activity in the supernatant decreased from day2 to day 6 (**Fig. 7A**). Next, the 288 ISGs were screened (**Fig. 7B**). More than a dozen ISG candidates which can inhibit HBV cccDNA activity to less than 33.3% were identified. Additionally, 3 ISG candidates which can enhance the HBV cccDNA activity to 300% were identified. These candidates are further analyzed.

[0122] Next, a similar strategy was used to screen a small epigenetic gene inhibitor library (73 inhibitors). HepG2 cells were first transfected with mcHBVcccDNA (**Fig. 8A**). The cells were incubated 3 days for mcHBVcccDNA to be epigenetically modified. Then, the chemical was used on duplicated plates. Results from a representative plate are shown (**Fig. 8B**).

[0123] The data above showed that mcHBVcccDNA can be applied for high through screening.

[0124] The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That which is claimed is:

1. A nucleic acid comprising a hepatitis B virus (HBV) genome encoding Core, PreS1/2/S, Pol, and X proteins, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the nucleotide sequence encoding the Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion.

2. The nucleic acid of claim 1, wherein the polynucleotide encoding the reporter protein is inserted about 30 to about 300 nucleotides downstream of the A of the start codon of the nucleotide sequence encoding the Core protein.

3. The nucleic acid of claim 1 or 2, wherein the polynucleotide encoding the reporter protein is inserted about 84 nucleotides downstream of the A of the start codon of the nucleotide sequence encoding the Core protein.

4. The nucleic acid of any one of claims 1-3, wherein a polynucleotide encoding N-coreOpt protein is inserted in-frame between the polynucleotide encoding the reporter protein and the nucleotide sequence encoding the C-terminal Core protein such that a full-length Core protein is encoded by the polynucleotide downstream of the polynucleotide encoding the reporter protein.

5. The nucleic acid of claim 4, wherein the polynucleotide encoding said N-coreOpt protein differs in nucleotide sequence from the polynucleotide encoding said N-terminal Core protein.

6. The nucleic acid of any one of claims 1-5, wherein the reporter protein is a luciferase.

7. The nucleic acid of claim 6, wherein the reporter protein is *Gaussia* luciferase.

8. The nucleic acid of any one of claims 1-7, further comprising a promoter operably linked to the HBV genome.

9. The nucleic acid of claim 8, wherein the promoter is a cytomegalovirus promoter.

10. The nucleic acid of any one of claims 1-9, wherein the nucleic acid is a plasmid.

11. The nucleic acid of any one of claims 1-10, wherein the HBV genome is from HBV genotype A, B, C, D, E, F, G, or H.

12. A replication competent and infectious HBV particle comprising the nucleic acid of any one of claims 1-11 or a HBV genome produced from the nucleic acid.

13. A method for producing replication competent and infectious HBV particles, comprising introducing the nucleic acid of any one of claims 1-11 into a mammalian cell under conditions suitable for HBV to replicate.

14. The method of claim 13, further comprising isolating the HBV particles.

15. The method of claim 13 or 14, wherein the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell line.

16. A method for producing replication competent and infectious HBV particles, comprising introducing the nucleic acid of any one of claims 1-11 into a non-human mammal.

17. A non-human mammal model for HBV infection, comprising a non-human mammal into which the nucleic acid of any one of claims 1-11 has been introduced.

18. A method for detecting protein expression from the nucleic acid of any one of claims 1-11 or a HBV genome produced therefrom, comprising detecting activity and/or an amount of the reporter protein, wherein detection of reporter protein activity and/or amount detects protein expression from the nucleic acid of any one of claims 1-11 or a HBV genome produced therefrom.

19. A method for identifying a compound that modulates replication of HBV, the method comprising contacting a cell comprising the nucleic acid of any one of claims 1-11 or a HBV genome produced therefrom with a compound, incubating the cell under conditions wherein HBV replicates in the cell in the absence of the compound; and detecting the replication level of the nucleic acid or the HBV genome produced therefrom, wherein a modulation in the replication level of the HBV genome in the cell in the presence of the compound compared to the replication level in a cell in the absence of the compound identifies the compound as a compound that modulates replication of HBV.

20. The method of claim 19, wherein detecting the replication level of the nucleic acid or the HBV genome produced therefrom comprises detecting reporter protein activity.

21. The method of claim 19 or 20, wherein the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell.

22. The method of claim 19 or 20, wherein the cell is in a non-human mammal.

23. A pre-minicircle vector for production of a hepatitis B virus (HBV) covalently closed circular DNA (cccDNA), said pre-minicircle vector comprising:

a HBV genome encoding Core, PreS1/2/S, Pol, and X proteins, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the nucleotide sequence encoding the HBV Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion; and

an intron comprising a prokaryotic origin of replication and a marker nucleotide sequence and surrounded by site-specific recombination sites inserted into the polynucleotide encoding the reporter protein.

24. The pre-minicircle vector of claim 23, wherein the vector is a plasmid.

25. The pre-minicircle vector of claim 23 or 24, wherein the intron further comprises a polynucleotide encoding a site-specific recombinase.

26. The pre-minicircle vector of any one of claims 23-25, wherein the marker nucleotide sequence is an antibiotic resistance gene.

27. The pre-minicircle vector of any one of claims 23-26, wherein the prokaryotic origin of replication is a high copy number origin of replication.

28. The pre-minicircle vector of claim 27, wherein the prokaryotic origin of replication is from plasmid pUC19.

29. The pre-minicircle vector of any one of claims 23-28, wherein the site-specific recombination sites are selected from the group consisting of: attB, attP, loxP sites, γδ res sites, FRT sites, hixL, hixR, TN3 res sites, Tn21 res sites, psi sites, cer sites, and any combination thereof.

30. The pre-minicircle vector of any one of claims 23-29, wherein the site-specific recombination utilizes an enzyme selected from the group consisting of: γδ resolvase, Hin recombinase, P1 Cre, yeast 2 micron Flp, Tn3 resolvase, Tn21 resolvase, λ integrase, XerCD, and any combination thereof.

31. The pre-minicircle vector of any one of claims 23-30, wherein the polynucleotide encoding a reporter protein is inserted about 30 to about 300 nucleotides downstream of the A of the start codon of the nucleotide sequence encoding the Core protein.

32. The pre-minicircle vector of any one of claims 23-31, wherein the polynucleotide encoding a reporter protein is inserted about 84 nucleotides downstream of the A of the start codon of the nucleotide sequence encoding the Core protein.

33. The pre-minicircle vector of any one of claims 23-32, wherein a polynucleotide encoding N-coreOpt protein is inserted in-frame between the polynucleotide encoding the reporter protein and the nucleotide sequence encoding the C-terminal Core protein such that a full-length Core protein is encoded by the polynucleotide downstream of the polynucleotide encoding the reporter protein.

34. The pre-minicircle vector of claim 33, wherein the polynucleotide encoding said N-coreOpt protein differs in nucleotide sequence from the polynucleotide encoding said N-terminal Core protein.

35. The pre-minicircle vector of any one of claims 23-34, wherein the reporter protein is a luciferase.

36. The pre-minicircle vector of claim 35, wherein the reporter protein is *Gaussia* luciferase.

37. The pre-minicircle vector of any one of claims 23-36, wherein the HBV genome is from HBV genotype A, B, C, D, E, F, G, or H.

38. A kit for the production of HBV cccDNA, comprising the pre-minicircle vector of any one of claims 23-37.

39. A minicircle HBV cccDNA, said minicircle HBV cccDNA comprising:

a HBV genome, wherein a polynucleotide encoding a reporter protein is inserted in-frame into the nucleotide sequence encoding the HBV Core protein to encode a N-terminal Core protein : reporter protein : C-terminal Core protein fusion;

wherein the minicircle HBV cccDNA is devoid of plasmid backbone DNA sequences.

40. The minicircle HBV cccDNA of claim 39, further comprising a splice residue resulting from site-specific recombination inserted into the polynucleotide encoding the reporter protein.

41. The minicircle HBV cccDNA of claim 39 or 40, wherein the polynucleotide encoding a reporter protein is inserted about 30 to about 300 nucleotides downstream of the A of the start codon of the nucleotide sequence encoding the Core protein.

42. The minicircle HBV cccDNA of any one of claims 39-41, wherein the polynucleotide encoding a reporter protein is inserted about 84 nucleotides downstream of the A of the start codon of the nucleotide sequence encoding the Core protein.

43. The minicircle HBV cccDNA of any one of claims 39-42, wherein a polynucleotide encoding N-coreOpt protein is inserted in-frame between the polynucleotide encoding the reporter protein and the nucleotide sequence encoding the C-terminal Core protein such that a full-length Core protein is encoded by the polynucleotide downstream of the polynucleotide encoding the reporter protein.

44. The minicircle HBV cccDNA of claim 43, wherein the polynucleotide encoding said N-coreOpt protein differs in nucleotide sequence from the polynucleotide encoding said N-terminal Core protein.

45. The minicircle HBV cccDNA of any one of claims 39-44, wherein the reporter protein is a luciferase.

46. The minicircle HBV cccDNA of claim 45, wherein the reporter protein is *Gaussia* luciferase.

47. The minicircle HBV cccDNA of any one of claims 39-46, wherein the HBV genome is from HBV genotype A, B, C, D, E, F, G, or H.

48. A cell comprising the minicircle HBV cccDNA of any one of claims 39-47.

49. The cell of claim 48, wherein the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell.

50. A kit for the study of HBV cccDNA, comprising the minicircle HBV cccDNA of any one of claims 39-47.

51. A method for the production of minicircle HBV cccDNA, comprising introducing the pre-minicircle vector of any one of claims 23-37 into bacteria to replicate the vector and culturing the bacteria under conditions suitable for site-specific recombination to occur, thereby producing minicircle HBV cccDNA.

52. The method of claim 51, wherein the bacteria express a site-specific recombinase.

53. The method of claim 51 or 52, further comprising isolating the minicircle HBV cccDNA.

54. A method for the production of detectable HBV cccDNA, comprising introducing the minicircle HBV cccDNA of any one of claims 39-47 into a mammalian cell under conditions suitable for transcription of the minicircle HBV cccDNA.

55. The method of claim 54, wherein the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell.

56. The method of claim 54, wherein the cell is in a mammal.

57. A method for detecting protein expression from HBV cccDNA, comprising introducing the minicircle HBV cccDNA of any one of claims 39-47 into a mammalian cell under conditions suitable for transcription of the minicircle HBV cccDNA and detecting activity and/or an amount of the reporter protein, thereby detecting expression of HBV cccDNA.

58. The method of claim 57, wherein the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell.

59. The method of claim 58, wherein measuring activity and/or amount of the reporter protein comprises measuring activity and/or amount in culture supernatant.

60. The method of claim 57, wherein the cell is in a non-human mammal.

61. The method of claim 60, wherein detecting activity and/or amount of the reporter protein comprises detecting activity and/or amount in a sample from the mammal.

62. A method for identifying a compound that modulates the amount and/or activity of HBV cccDNA, the method comprising contacting a cell comprising the minicircle HBV cccDNA of any one of claims 39-47 with a compound, incubating the cell under conditions wherein the minicircle HBV cccDNA is expressed to produce reporter protein in the absence of the compound; and detecting activity and/or amount of the reporter protein, wherein a change in reporter protein activity and/or amount in the cell in the presence of the compound compared to the reporter protein activity and/or amount in a cell in the absence of the compound identifies the compound as a compound that modulates the level and/or activity of HBV cccDNA.

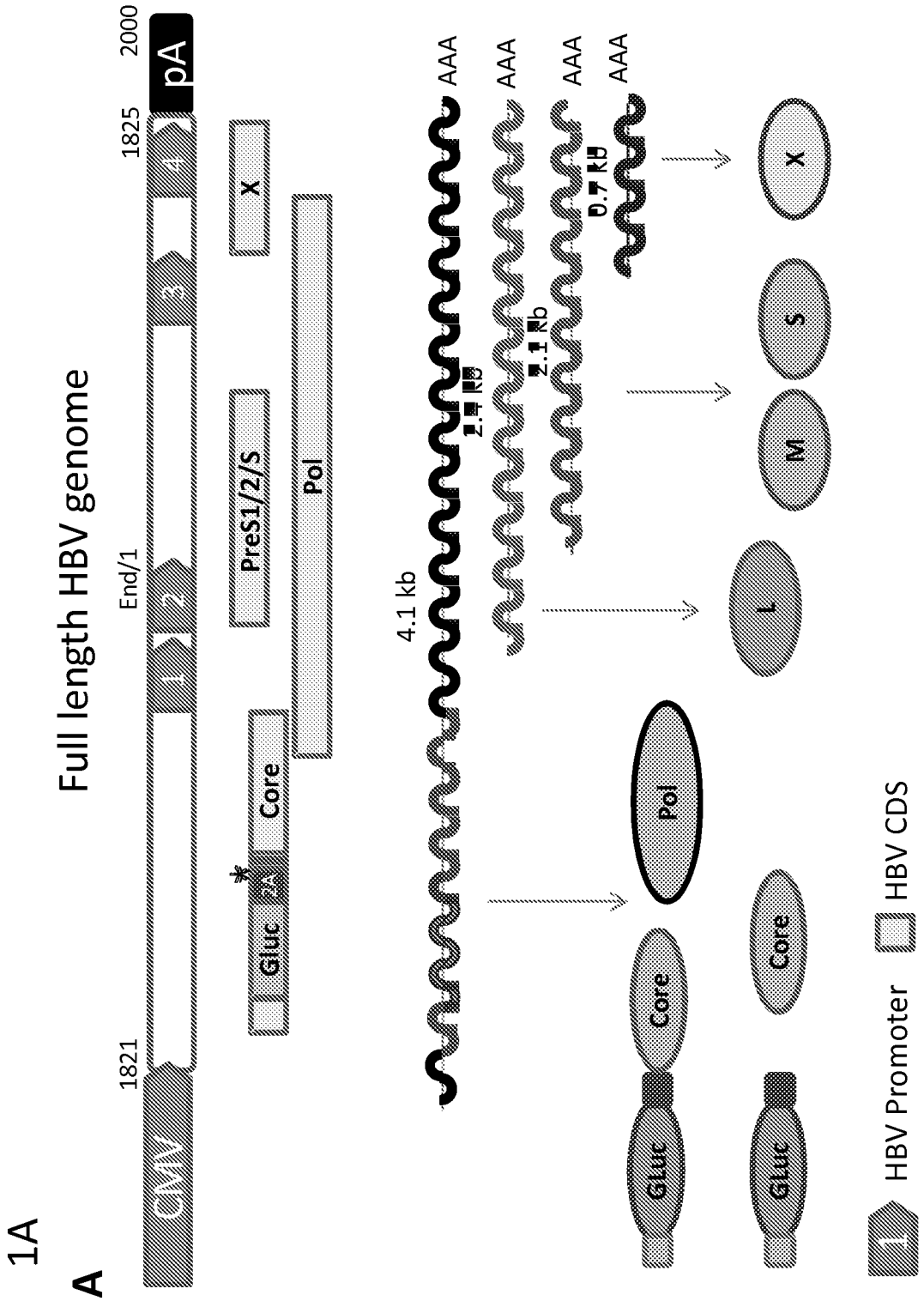
63. The method of claim 62, wherein the cell is a primary human hepatocyte, human hepatoma cell, or other hepatocyte-derived cell.

64. The method of claim 62 or 63, wherein detecting activity and/or amount of the reporter protein comprises detecting activity and/or amount in culture supernatant.

65. The method of claim 62, wherein the cell is in a non-human mammal.

66. The method of claim 65, wherein detecting activity and/or amount of the reporter protein comprises detecting activity and/or amount in a sample from the mammal.

Fig. 1A


A
Full length HBV genome
1821
End/1
1825
2000
CMV
1
2
3
4
pA
Gluc
2A
Core
PreS1/2/S
Pol
X
4.1 kb
2.4 Kb
2.1 Kb
0.7 Kb
AAA
Gluc
Core
Pol
Core
L
M
S
X
HBV Promoter
HBV CDS

FIGS. 1B-1D

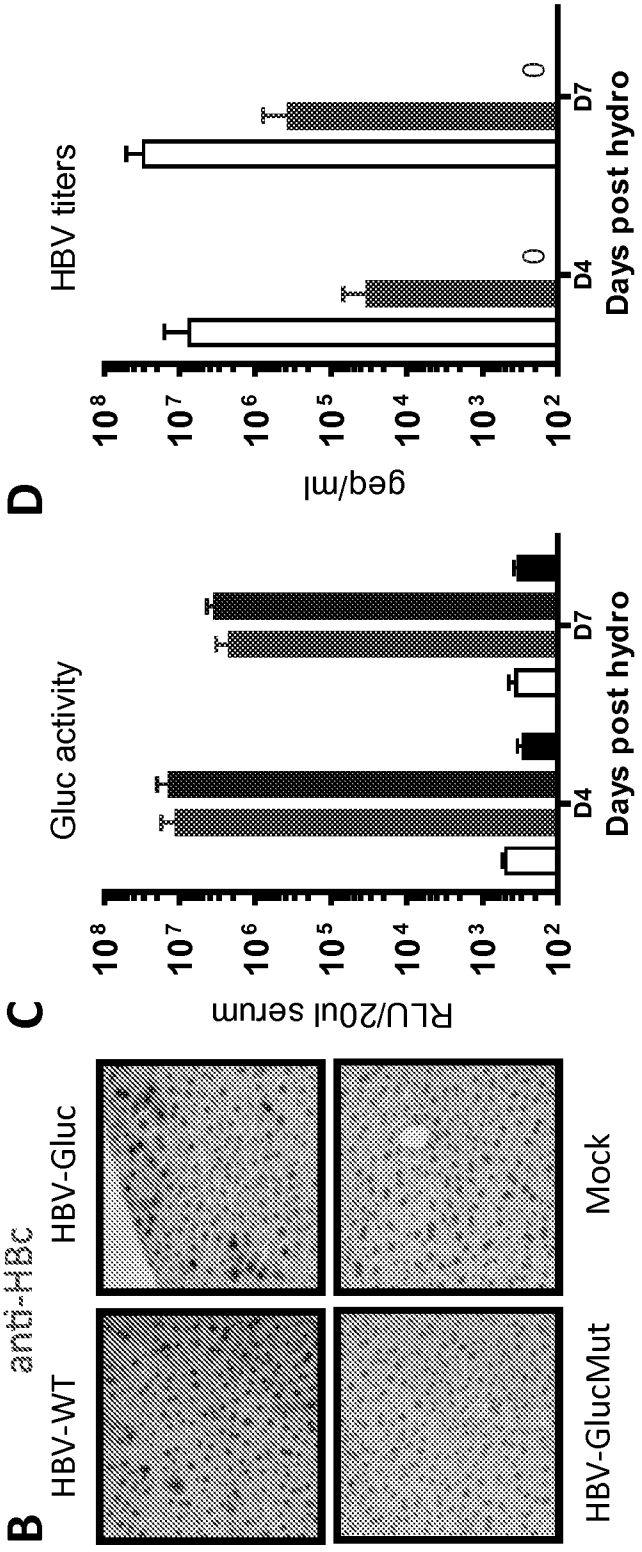

B
anti-HBc
HBV-WT
HBV-Gluc
HBV-GlucMut
Mock
C
Gluc activity
RLU/20ul serum
Days post hydro
D4
D7
D
HBV titers
geq/ml
Days post hydro
D4
D7
0
0

FIG. 2A

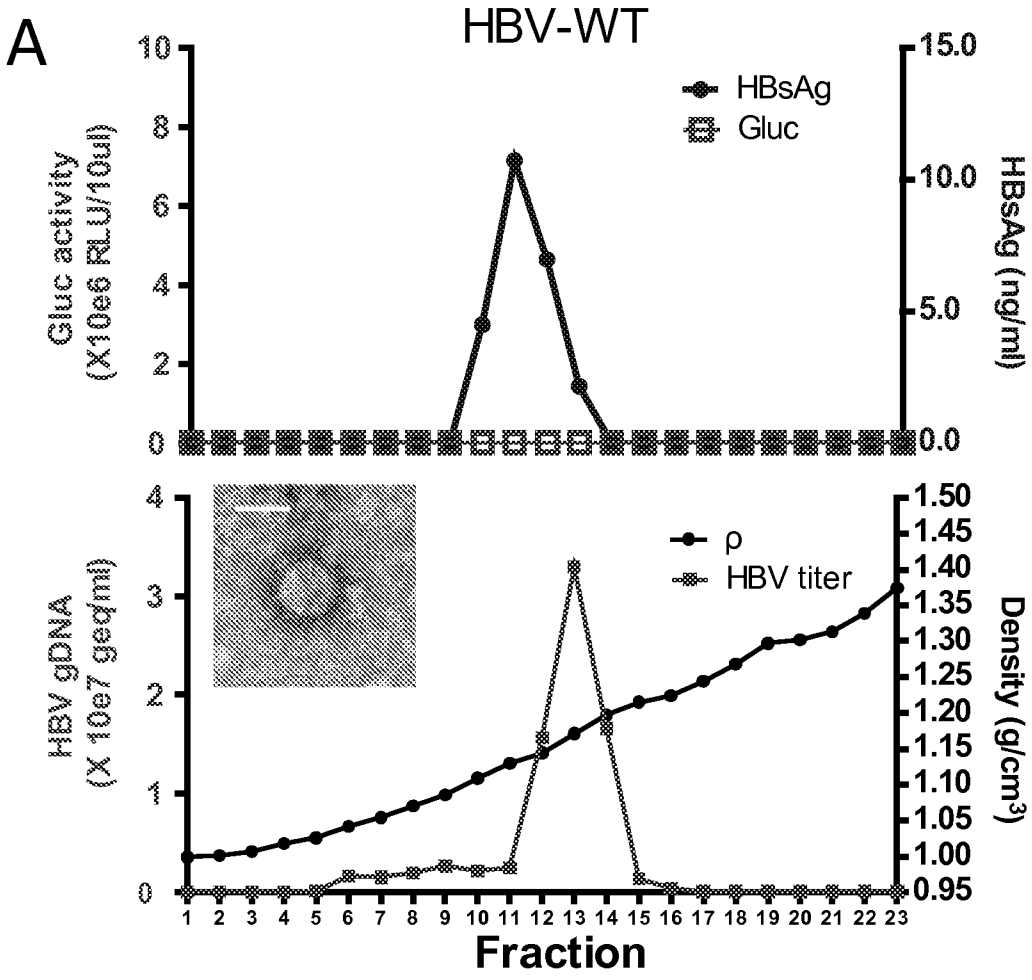

A
HBV-WT
HBsAg
Gluc
Gluc activity (X10e6 RLU/10ul)
HBsAg (ng/ml)
ρ
HBV titer
HBV gDNA (X 10e7 geq/ml)
Density (g/cm3)
Fraction

FIG. 2B

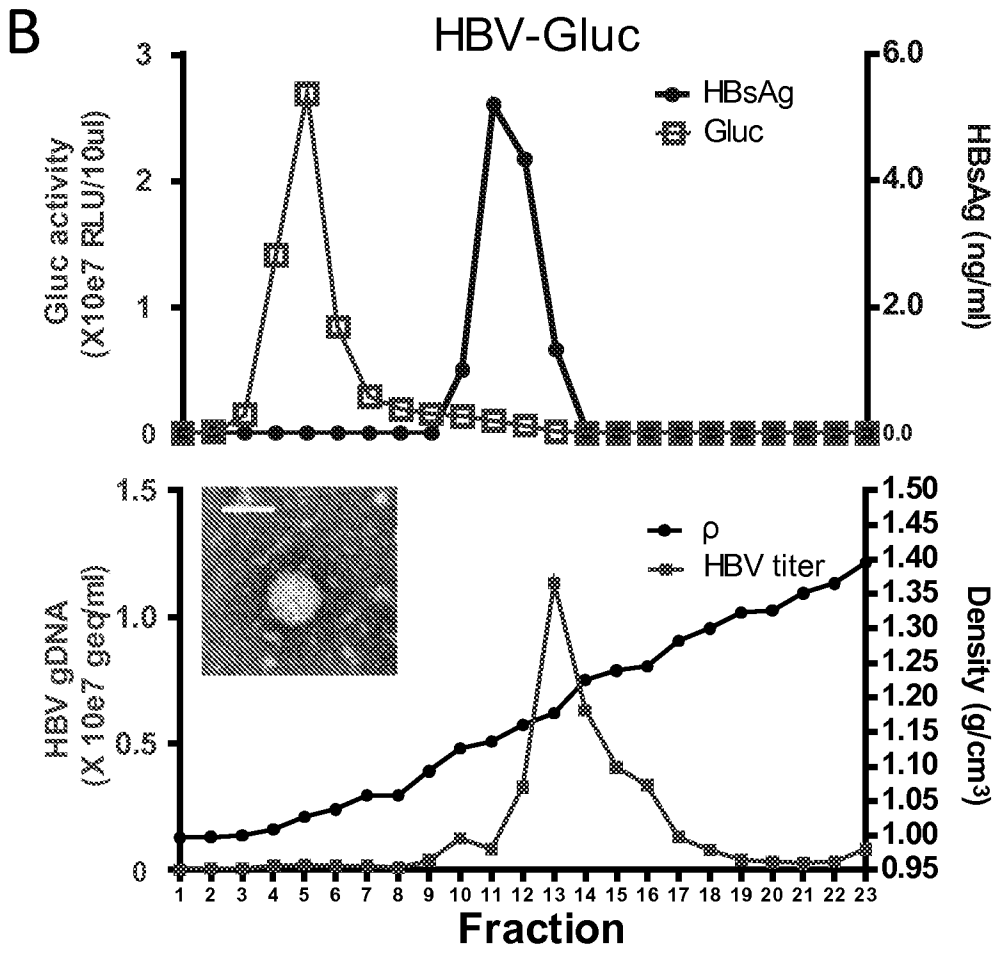
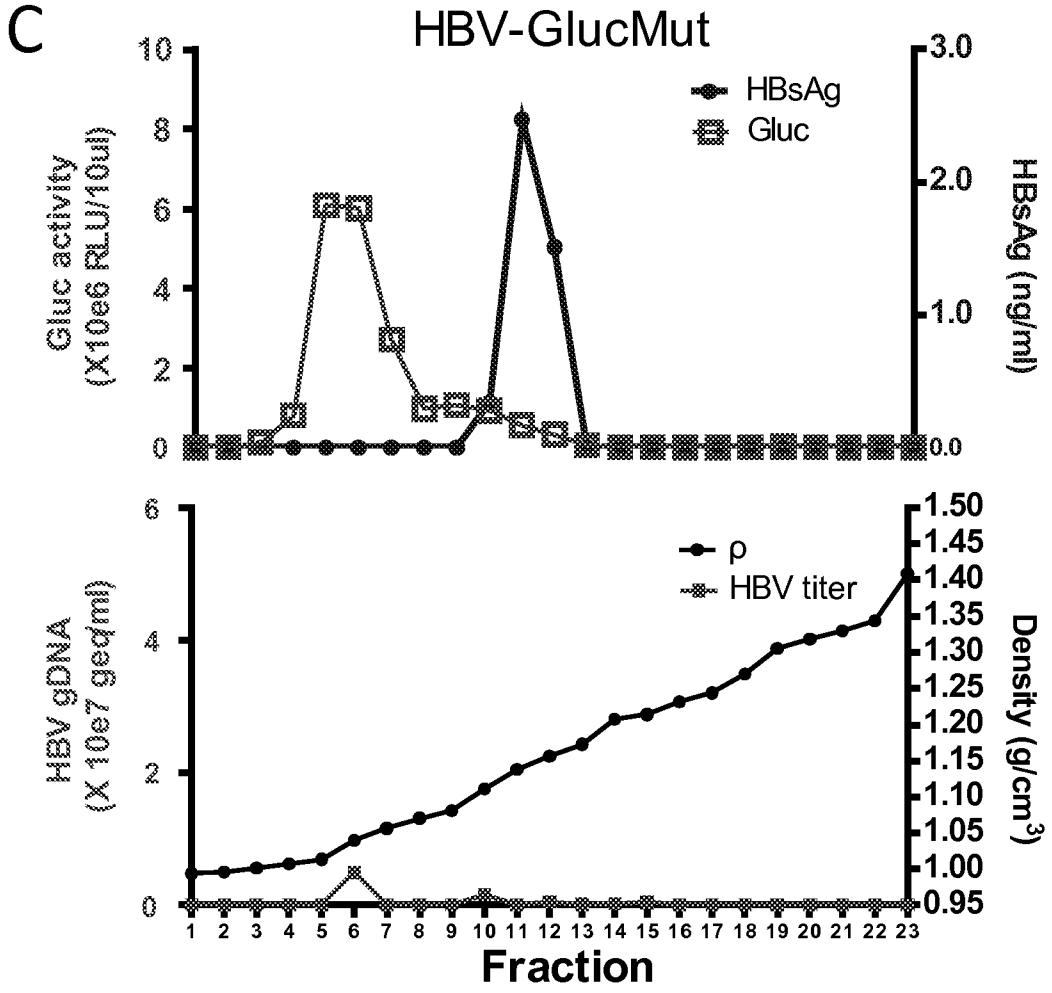

B
HBV-Gluc
HBsAg
Gluc
Gluc activity (X10e7 RLU/10ul)
HBsAg (ng/ml)
ρ
HBV titer
HBV gDNA (X 10e7 geq/ml)
Density (g/cm3)
Fraction

FIG. 2C


C
HBV-GlucMut
HBsAg
Gluc
Gluc activity (X10e6 RLU/10ul)
HBsAg (ng/ml)
ρ
HBV titer
HBV gDNA (X 10e7 geq/ml)
Density (g/cm3)
Fraction

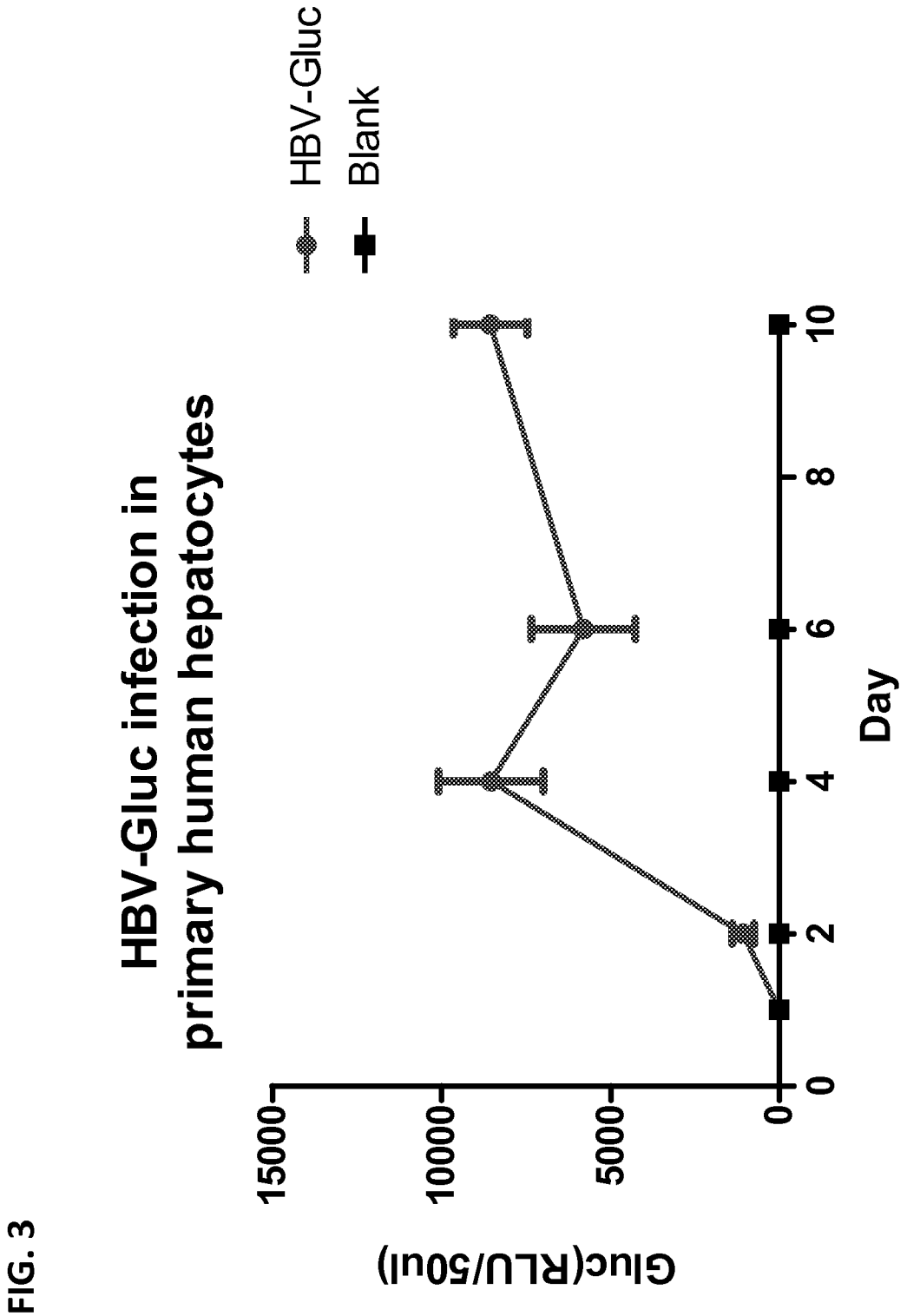
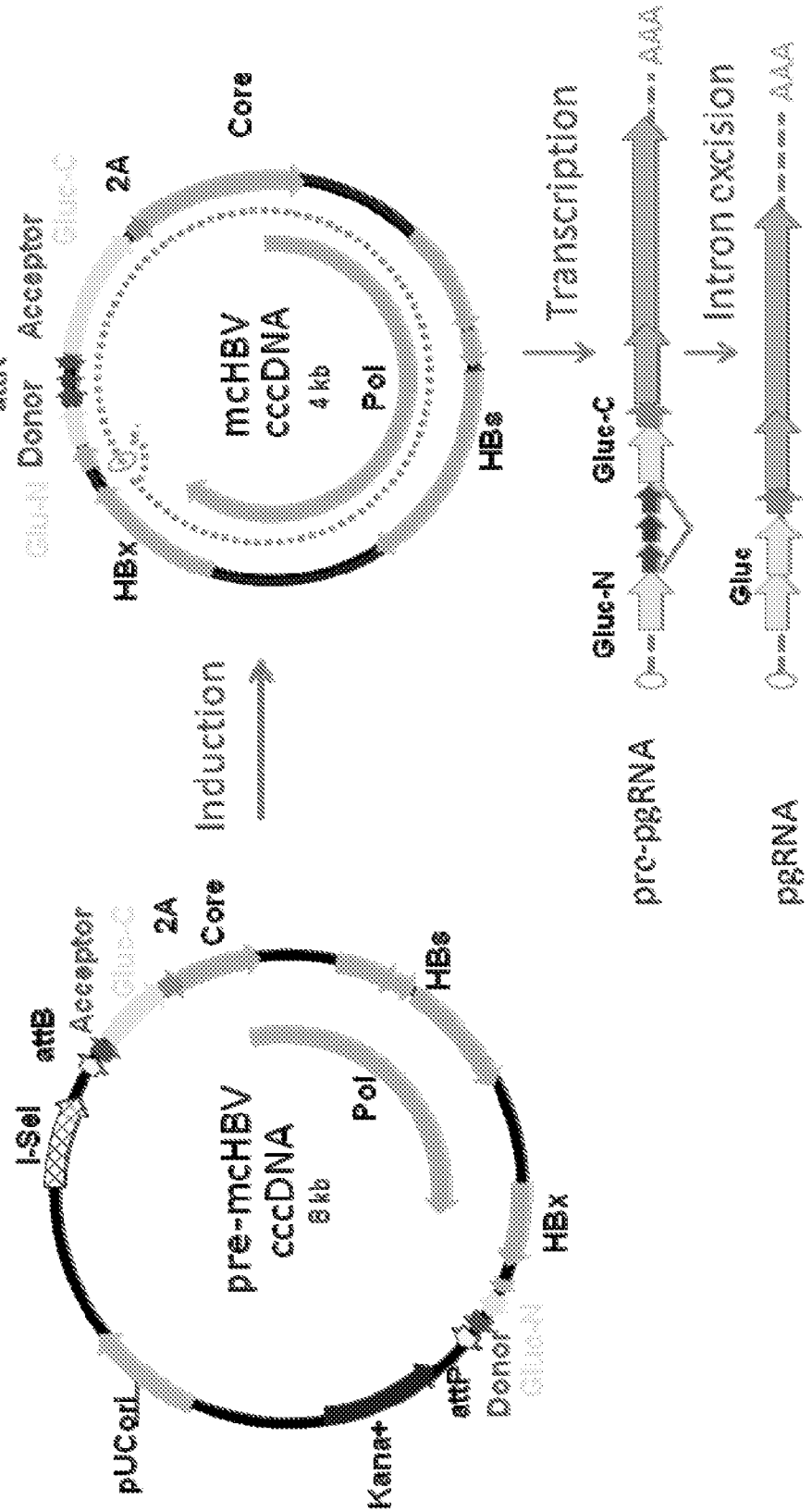

HBV-Gluc infection in primary human hepatocytes
HBV-Gluc
Blank
Gluc(RLU/50ul)
15000
10000
5000
0
0
2
4
6
8
10
Day

FIG. 3

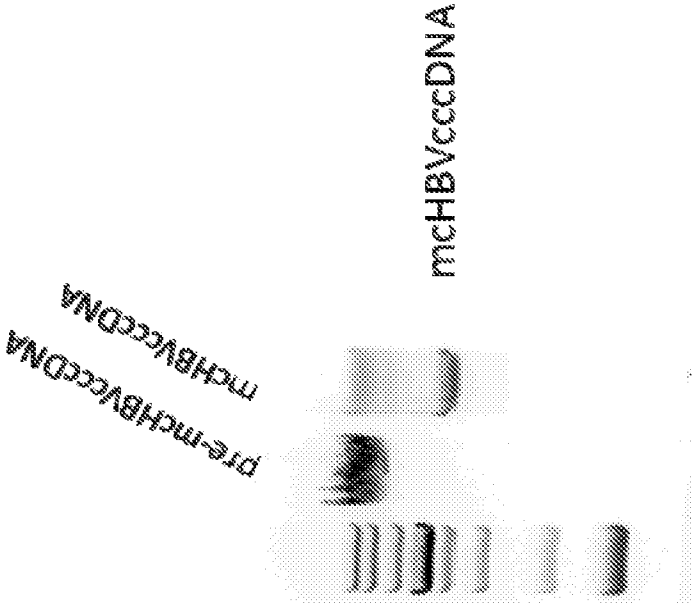
FIG. 4A

A

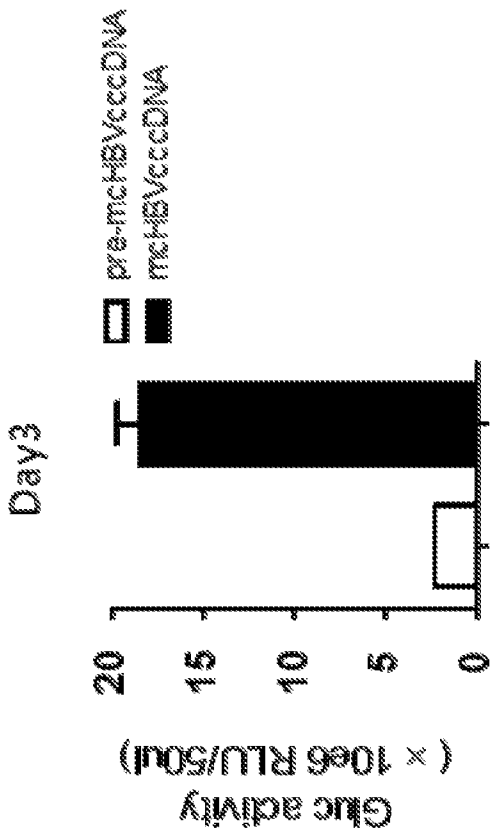

pre-mcHBV
cccDNA
8 kb
I-SceI
attB
Acceptor
Gluc-C
2A
Core
HBs
Pol
HBx
Kana+
attP
Donor
Gluc-N
mcHBV
cccDNA
4 kb
attR
Donor
Acceptor
Gluc-C
2A
Core
HBs
Pol
HBx
Induction
Transcription
Intron excision
Gluc-N
Gluc-C
pre-pgRNA
AAA
Gluc
pgRNA
AAA

FIGS. 4B-4C

B


pre-mcHBVcccDNA
mcHBVcccDNA
mcHBVcccDNA

C


Day3
pre-mcHBVcccDNA
mcHBVcccDNA
20
15
10
5
0
Gluc adinity
(× 10e6 RLU/50ul)

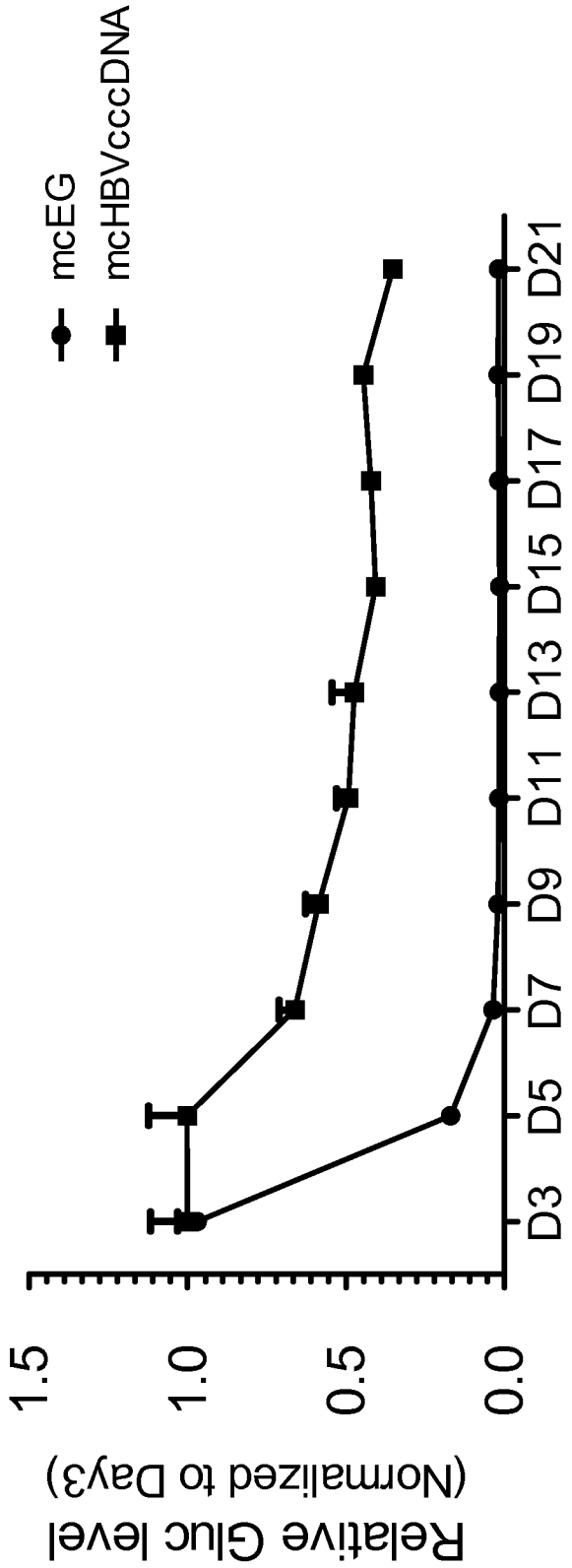

mcEG
mcHBVcccDNA
Relative Gluc level
(Normalized to Day3)
1.5
1.0
0.5
0.0
D3
D5
D7
D9
D11
D13
D15
D17
D19
D21

FIG. 5

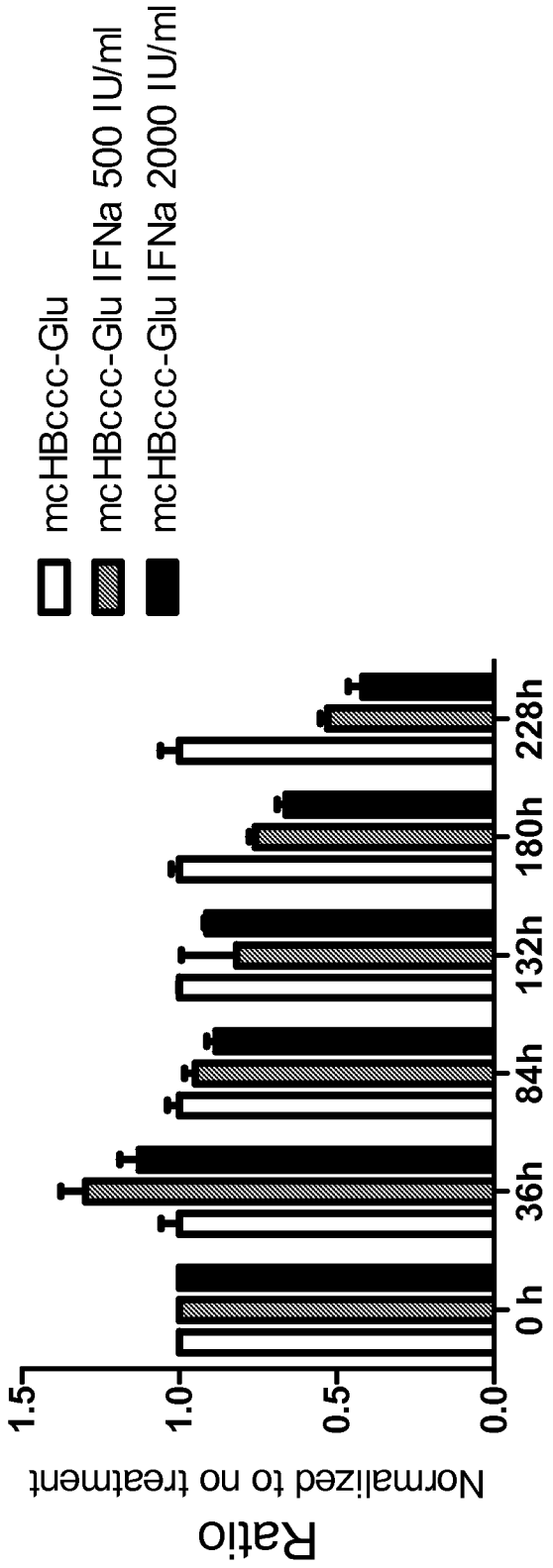

HBV_IFN
mcHBccc-Glu
mcHBccc-Glu IFNa 500 IU/ml
mcHBccc-Glu IFNa 2000 IU/ml
Ratio
Normalized to no treatment
1.5
1.0
0.5
0.0
0 h
36h
84h
132h
180h
228h

FIG. 6

FIGS. 7A-7B

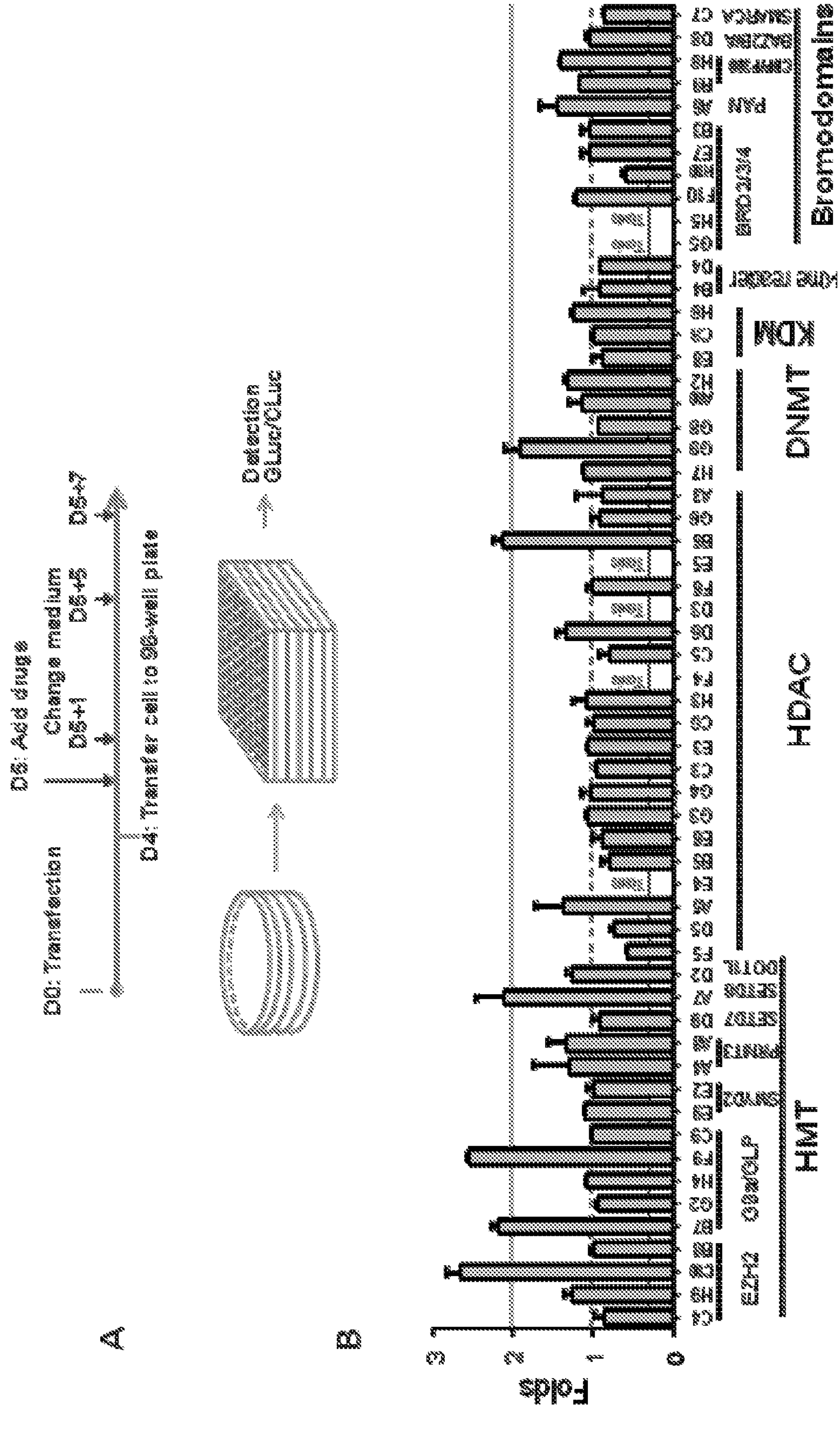

8 : 1
ISG
mcHBV
cccDNA
rM-ZAP
Control
rM-ZAP
Fold change in hepatocytes vs hepatomas
1.5
1.0
0.5
0.0
Day2
Day4
Day8

A

cccDNA activity (% of control)
300
100
30
10
Day1
Day2
Day4

B

FIG. 8


A
D0: Transfection
D4: Transfer cell to 96-well plate
D5: Add drugs
Change medium
D5+1
D5+5
D5+7
Detection
GLuc/CLuc
B
Folds
0
1
2
3
EZH2
G9a/GLP
SMYD2
PRMT3
SETD7
SETD8
DOT1L
HMT
HDAC
DNMT
KDM
Kme reader
BRD2/3/4
PAN
BAZ2B
SMARCA
Bromodomains

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/058256

A. CLASSIFICATION OF SUBJECT MATTER

(see extra sheet)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 15/36, 15/62 , 15/63 7/01, 5/10, C12P 21/00, 1/04, C12Q 1/68, 1/66

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CIPO, DEPATISNET,DWPI,EAPATIS,EMBL,EPO-Internal,ESP@CE, ESP@CENET, Google, KIPRIS,PAJ, PabMED, RUPTO, SCIENCEDIRECT, SIPO,USPTO,WIPO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	PETER A. KRATZ et al. Native display of complete foreign protein domains on the surface of hepatitis B virus capsids, Proc. Natl. Acad. Sci. USA, March 1999, Vol. 96, pp. 1915-1920	1-3, 12 13-25, 48-66
Y	US 2003/0175863 A1 (ASHLEY BIRKETT J.) 18.09.2003, paragraph [0150], claims	13-16
Y	ZIHUA WANG et al. Replication-Competent Infectious Hepatitis B Virus Vectors Carrying Substantially Sized Transgenes by Redesigned Viral Polymerase Translation, PLOS ONE, April 2013, Vol. 8, Issue 4, e60306, pp.1-14	15, 21, 49, 55, 58, 63
Y	SHENG-QIANG LIANG et al. A Mouse Model for Studying the Clearance of Hepatitis B Virus In Vivo Using a Luciferase Reporter, PLOS ONE, April 2013, Vol. 8, Issue 4, e60005, pp.1-9	17-22, 54-66

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 January 2016 (13.01.2016)	11 February 2016 (11.02.2016)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer O.Fedonova Telephone No. 495 531 65 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2015/058256

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MARK A KAY et al. A robust system for production of minicircle DNA vectors, Nature Biotechnology, 2010, Vol. 28, no. 12, pp.1287- 1291	17-25, 48-66

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2015/058256

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-11, 26-37, 42-47
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

C12N 15/36 *(2006.01)*
C12N 15/62 *(2006.01)*
C12N 7/01 *(2006.01)*
C12P 21/00 *(2006.01)*
C12P 1/04 *(2006.01)*
C12Q 1/68 *(2006.01)*
C12Q 1/66 *(2006.01)*
C12N 15/63 *(2006.01)*
C12N 5/10 *(2006.01)*