Supporting Information for

N6-Methyladenosine Modification of Hepatitis B Virus RNA Differentially Regulates The Viral Life Cycle

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Supplementary text (Materials and methods).

Figures S1-S3.

References for SI reference citations.

Supplementary Information Text:

Methods and Materials

Plasmids and antibodies. pHBV 1.3mer was a kind gift from Dr. Wang-Shik Ryu and obtained from the Addgene (1, 2). Anti-METTL3 antibody was purchased from Proteintech (USA), anti-METTL14 and anti-ALKBH5 were from Sigma (USA), anti-FTO and anti-YTHDF2 were from Abcam (USA), anti-Actin and anti-FLAG antibodies were from Cell Signaling Technologies (USA), anti-GAPDH, anti-HBsAg, anti-HBcAg (a kind gift from Dr. Haitao Guo, Indiana University, IN, USA), anti-YTHDF3 and anti-Rabbit IgG HRP antibodies were from Santa Cruz Biotechnology (USA), anti-mouse IgG HRP antibody from Promega (USA) and anti-m^6A antibody was obtained from Synaptic Systems (Germany).

Cell culture and transfection. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and HepAD38 cells were maintained in RPMI 1640 with 20% FBS. Plasmids were transfected into HepG2 cells using Mirus TransIT-LT1 reagent (Mirus, USA) according to the manufacturer's protocol. For siRNA-mediated knockdown of METTL3, METTL14, FTO, AlkBH5, YTHDF2 and YTHDF3, siGENOME Human METTL3 (56339) siRNA-SMARTpool, siGENOME Human METTL14 (57721) siRNA-SMARTpool, siGENOME Human FTO (79068) siRNA-SMARTpool, siGENOME Human ALKBH5 (54890) siRNA-SMARTpool, siGENOME Human YTHDF2 (51441) siRNA-SMARTpool and siGENOME Human YTHDF3 (253953) siRNA-SMARTpool (Dharmacon, USA) were used; the ON-TARGET plus non-targeting pool (D-001810-10-05) was used as the scrambled control. siRNA was transfected using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, USA) according to the manufacturers protocol.

Quantitative PCR. RNA was isolated using Trizol reagent (Life Technologies, USA). m^6A RNA was immunoprecipitated according to the protocol described by Dominissini et al, 2013 (3). SuperScript III - First strand synthesis supermix (Thermo Fisher, USA) was used to prepare cDNA and quantitative PCR was done with 2X Dynamo SYBRgreen qRT-PCR master mix (Thermo Scientific, USA) using the following primers: HBV-specific primer forward, 5′-CTCAATCTCGGGAATCTCAATGT -3′, HBV-specific primer reverse, 5′-TGGATAAAACCTGGCAGGCATAAT -3′, HBV 3' UTR forward 5'-GTCAACGACCGACCTTG -3', HBV 3' UTR reverse 5'-

TGATTAGGCAGAGGTGAAAAAG-3' GAPDH forward, 5′-TGCACCACCAACTGCTTAGC-3′, GAPDH reverse, 5′-GGCATGGACTGTGGTCATGAG-3′. The RT-qPCR program was 95˚C for 10 min followed by 40 cycles at 94˚C for 20 sec, 55˚C for 30 sec, 72˚C for 30 sec, and fold changes in gene expression were calculated by the ΔΔCT method. For HBV DNA PCR, PrimeTime Gene expression mastermix was used (Integrated DNA Technologies, USA) with Taqman probe. The qPCR program was 95˚C for 3 min followed by 40 cycles of 95˚C for 30 sec and 60˚C for 45 sec.

MeRIP-Seq. For MeRIP seq, three independent experiments were conducted. PolyA RNA was purified from total RNA by using polyA Spin mRNA isolation kit (New England Biolabs, USA). Samples were fragmented using the Ambion RNA fragmentation reagent and purified by ethanol precipitation. Fragmented RNA was heated to 75˚C for 5 min, placed on ice for 3 min, and then incubated with anti-m^{6}A antibody (5 µg; Synaptic Systems, #202003) conjugated to Protein G Dynabeads (30 uL; Thermo Fisher Scientific) in MeRIP buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40) overnight at 4˚C. Beads were then washed 5 times with MeRIP buffer, and bound RNA was eluted in MeRIP buffer containing 6.7 mM m^{6}A sodium salt (Sigma). Eluted RNA was purified with the RNeasy mini kit (Qiagen) and concentrated by ethanol precipitation. Sequencing libraries were prepared from this RNA, as well as input RNA, using the TruSeq RNA sequencing (RNA-seq) kit (Illumina). Libraries were sequenced to 1 x 50 base-pair reads on the Illumina HiSeq2500 at the Weill Cornell Medicine Epigenomics Core Facility. Reads were aligned to a combined human (hg38) and viral reference genome using Spliced Transcripts Alignment to a Reference (STAR). We noted coverage for the entire virus was increased in IP samples with respect to input by approximately fivefold, but only a single peak remained after normalization. Mean coverage was plotted for all three replicates using CovFuzze (https://github.com/al-mcintyre/covfuzze). MeRIP qRT-PCR followed the same protocol, except that total RNA was not fragmented. Eluted RNA was reverse transcribed into cDNA and subjected to RT-qPCR.

Western blot assays. Cell pellets were washed twice with ice-cold PBS and lysed with 1X SDS protein loading buffer (50 mM Tris, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.1% bromophenol blue). The samples were boiled at 95˚C for 10 min and clarified lysates were resolved by SDS-10%-PAGE and transferred to nitrocellulose membranes (BioRad, USA). The

membrane was blocked with 5% BSA for 1 hr, followed by overnight incubation with primary antibodies (1:1000) diluted in 2% BSA. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 1 hour. The signals were detected using a chemiluminescence substrate (Millipore, USA). The NIH Image J software was used for blot densitometry.

Isolation of core particles. HBV core particles were isolated according to the protocol described by Belloni et al, 2009 (4). Transfected HepG2 cell plate (10 cm) was washed with PBS. Freshly prepared 1 ml of transfection lysis buffer (50 mM Tris-HCL pH8.0, 1 mM EDTA and 1% NP40) with Protease inhibitor cocktail was added to the plate and kept in 37˚C for 10 minutes. Then the lysate was transferred to 1.5 ml centrifuge tube. After mixing briefly the lysates were centrifuged for 1 minute at 14000 rpm. Then 5 mM $CaCl_2$ and 75 U micrococcal nuclease were added to the supernatant and incubated for 45 minute. After a brief centrifugation, 75 U micrococcal nuclease was added to the supernatant again and incubated for 45 minute in a 37˚C rotator. After a centrifugation for 1 minute at 14000 rpm supernatant was transferred to a new microcentrifuge tube and 32 µl of 0.5M EDTA and 260 µl of 35% PEG in 1.75M NaCl was added and kept in 4˚C for 1 hour. After a centrifugation at 13,000 rpm for 5 minute at 4°C, supernatant was discarded and the pellet was resuspended in 300 µL TNE buffer.

Site directed mutagenesis. HBV 1.3mer plasmid was used for mutagenesis experiment. Site directed mutagenesis kit was purchased from New England Biolabs and all the primers were designed by the NEBaseChanger tool (http://nebasechanger.neb.com). All the reaction conditions and the steps were according to the manufacturer's instructions.

Data Deposition. The raw data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE114486 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114486).

SI Figure Legends

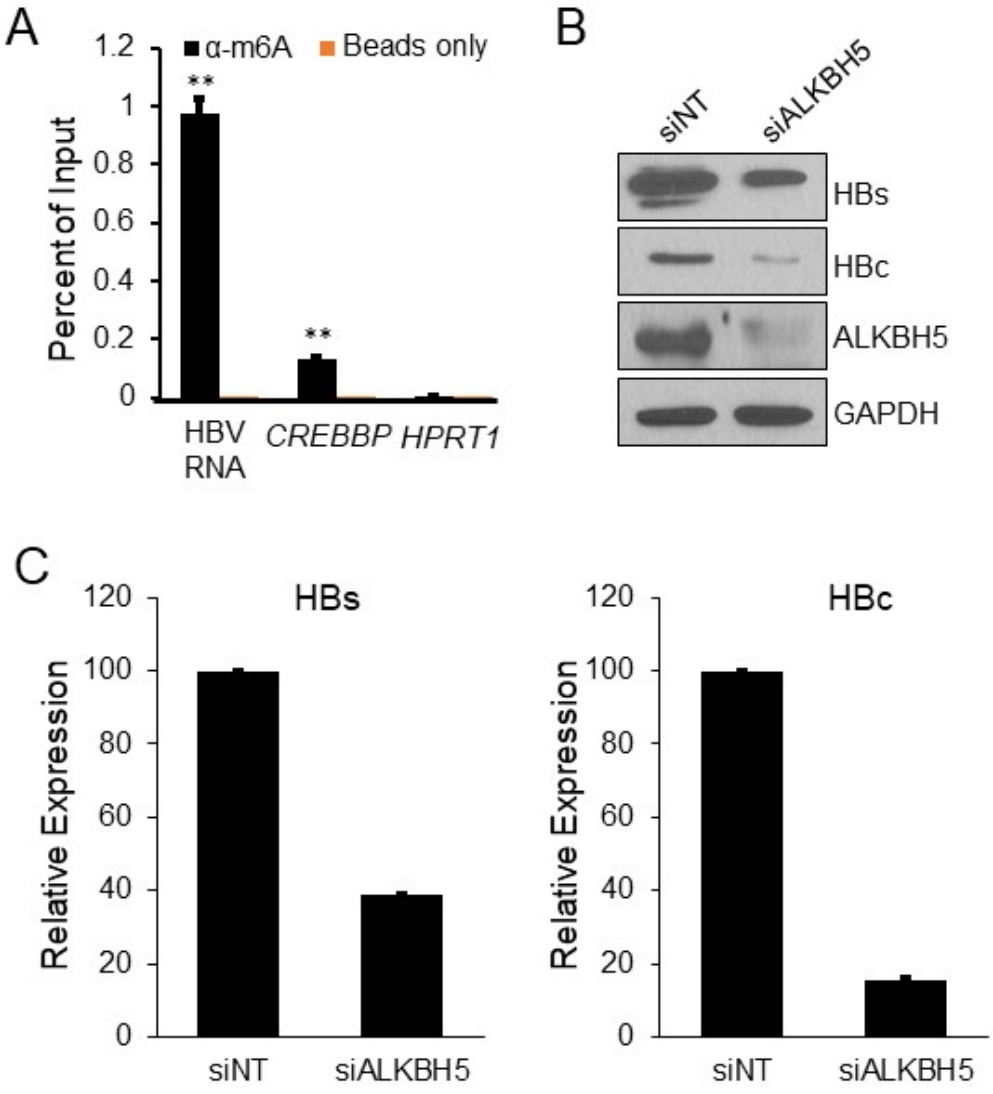
Fig. S1. HBV transcripts are m6A-modified. (A) MeRIP-RT-qPCR analysis of m^6A-modified HBV transcripts in HBV expressing HepG2 cells using 5'UTR HBV specific primers. The fraction of m^6A-modified RNA was calculated as the percent of the level present in the eluate compared to the total input RNA. (B) HepG2 cells siRNA depleted for ALKBH5 or non-targeting control (NT) were co-transfected with HBV 1.3mer, and after 5 days cellular lysates were analyzed for HBs and

HBc protein expression by immunoblotting. (C) The level of HBV proteins was quantified by Image J software. Data are from two independent experiments and the bars represent the mean ±SD. **p≤0.01

Fig. S2. The adenosine residues in the HBV epsilon stem loop. (A) Schematic showing the location of the epsilon stem loop in HBV transcripts. In pgRNA, the epsilon stem loop is present at both the 5' and 3' termini, while in all other transcripts it is only present at the 3' terminus. The primary sequence and the secondary structure of the stem loop is also shown. The adenosines shown in red represent the evolutionarily conserved residues across the various HBV genotypes identified by sequence alignment. Among all conserved-adenosines (outlined red), only A1907 is present within an m^6A consensus DRACH motif. (B) The HBV sequences of the epsilon stem loop (nt1820 to nt1907) of the following genotypes, with GenBank accession numbers indicated here, are shown: HBV-E, X75657; HBV-D, X02496; HBV-Cc, GQ205441; HBV-C, AB033556; HBV-Bc, GQ205440; HBV-Ba, D00330; HBV-Afr, AF297621; HBV-H, AY090454; HBV-F, X69798; HBV-Bj, AB073858; HBV-A, X02763; HBV ayr, X04615; Ch-109, AF222322; Gor-97, AJ131567; HBV-aus, A048704; WM-HBV, A226578; WHV, J02442; HBV- X02, JX978431. Solid dots denote homology, red squares denote mismatch and vertical lines denote gaps. The stars indicate the position of the conserved DRACH motif.

Fig. S3. HBV transcripts, including pgRNA, encoded by HBV 1.3mer plasmid. (A) The circular HBV genome, as well as the overlapping coding sequence and the terminal redundancy of the HBV transcripts, are shown. During the first round of pgRNA transcription, the polyadenylation signal (which is few base pairs downstream to the transcription start site) is ignored by the polymerase, resulting in the pgRNA being 300 nt longer than the HBV genome and containing epsilon stem loops at both the termini. The HBV 1.3mer expression plasmid, described previously (1, 2), is designed to maintain this terminal redundancy of pgRNA during transcription. Therefore, it contains an HBV genomic sequence that is nearly 1.3 times longer than the native HBV genome. The restriction sites and their positions are depicted on the plasmid map and used for subsequent cloning. (B) The DRACH motif at lower stem of epsilon stem loop was mutated (A1907C) within the HBV 1.3mer plasmid.

SI Fig. S1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0.6
0.4
0.2
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HPRT1
B
siNT
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HBs
HBc
ALKBH5
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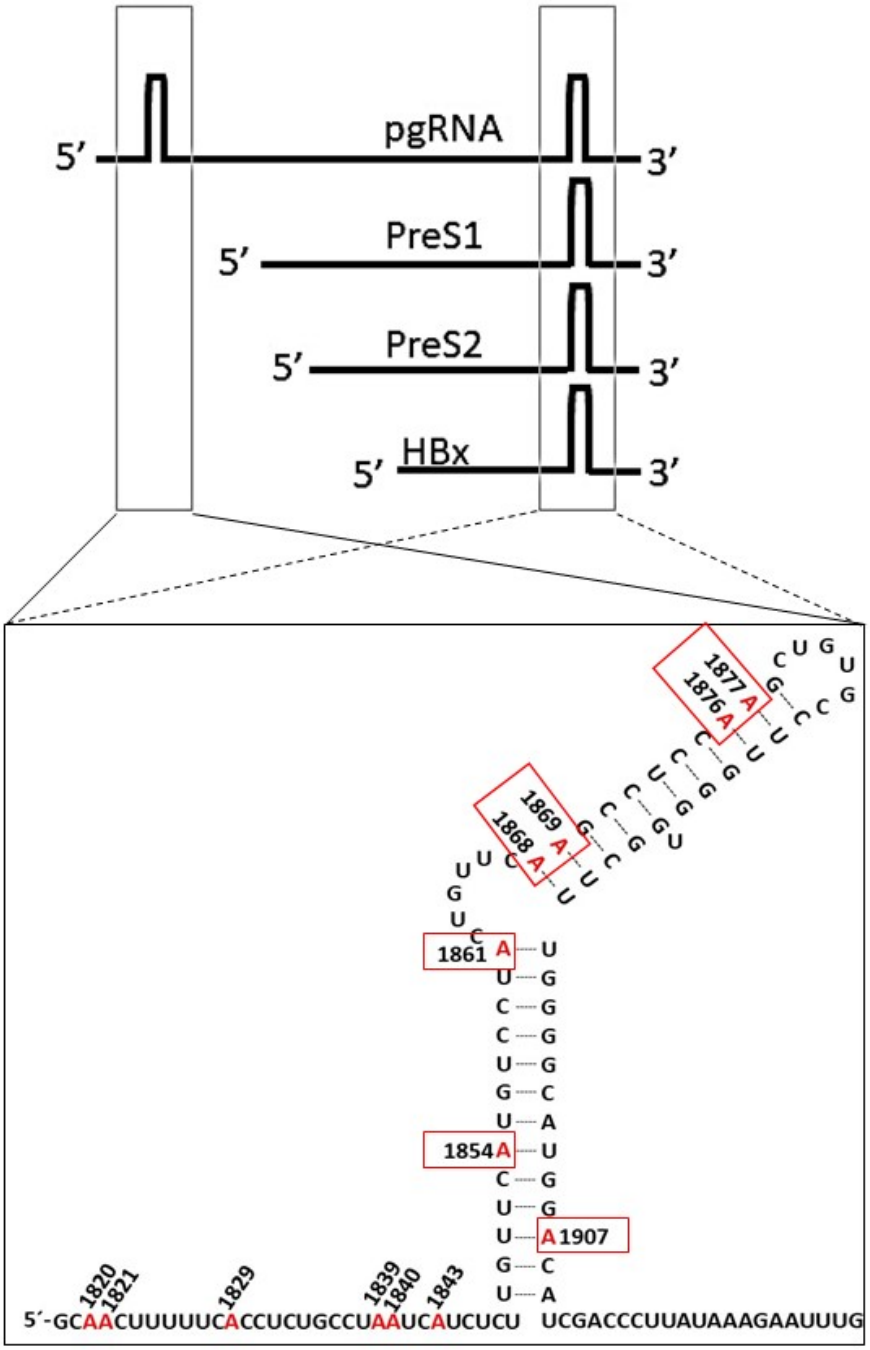
SI Fig. S2A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3′
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1861
1854
1907
1820
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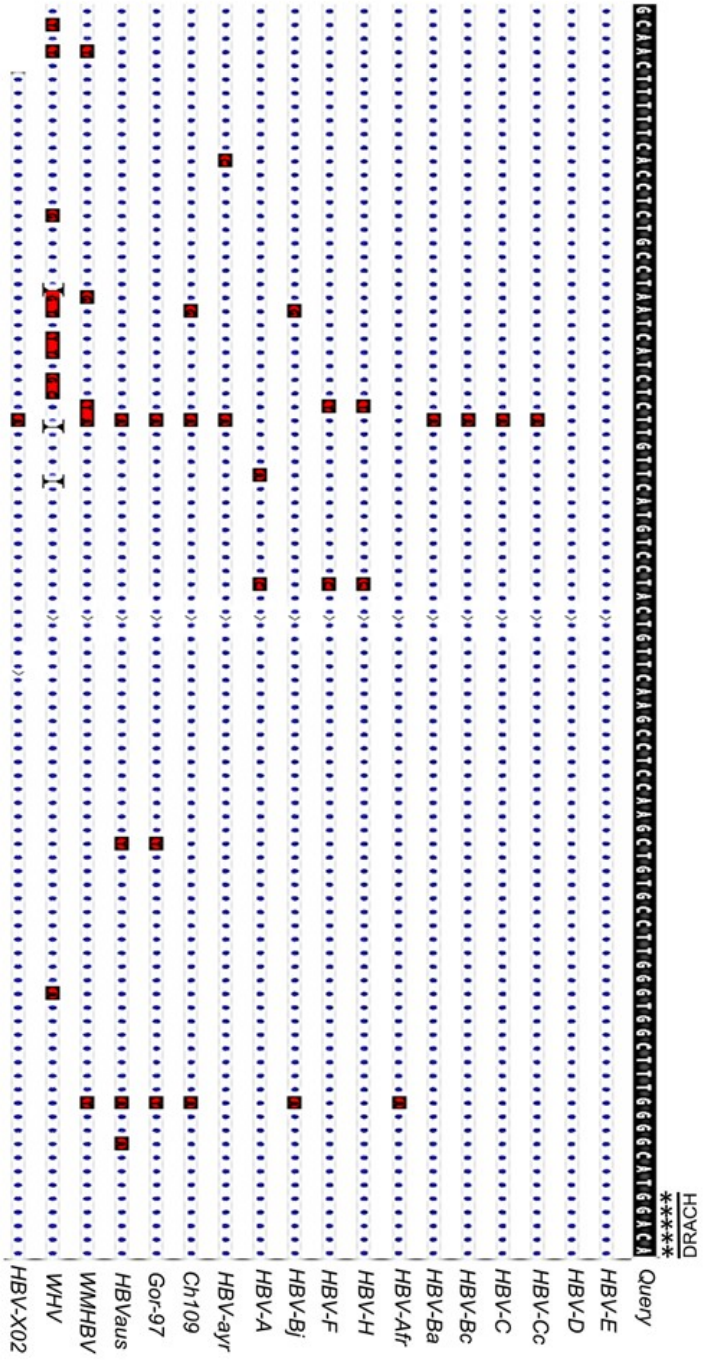
SI Fig. S2B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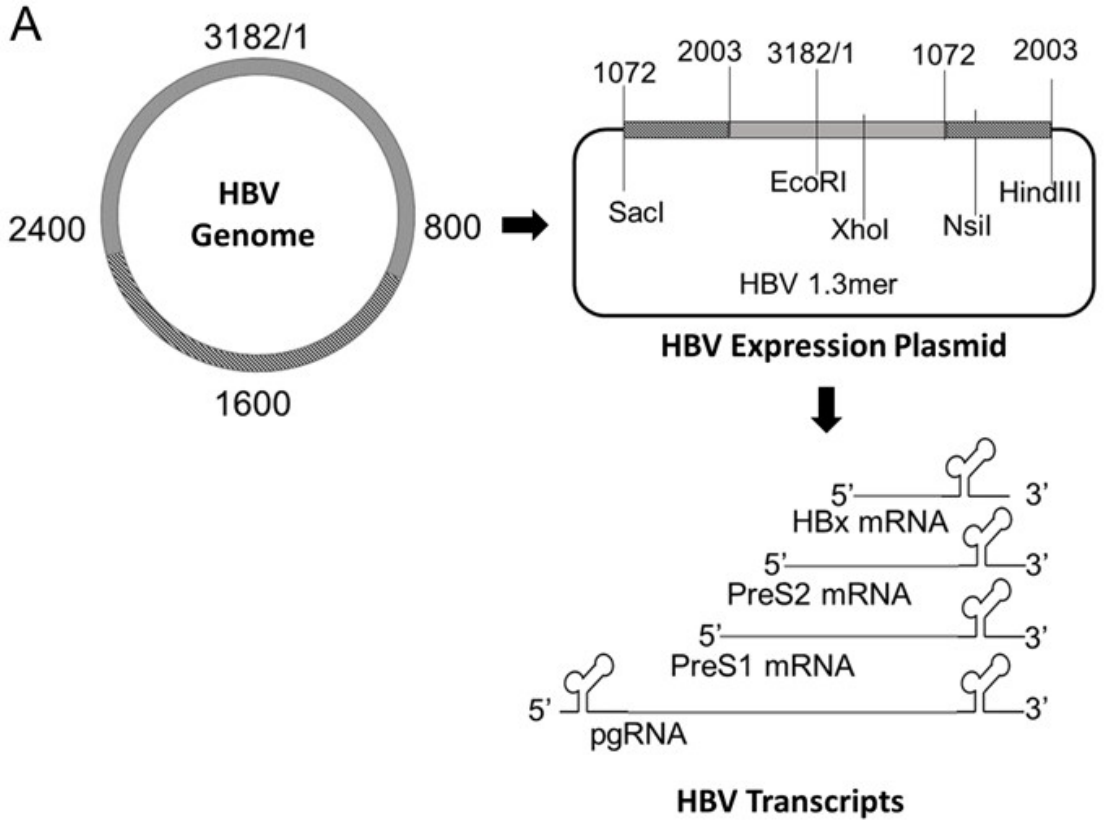
WMHBV
WHV
HBV-X02
DRACH

SI Fig. S3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SacI
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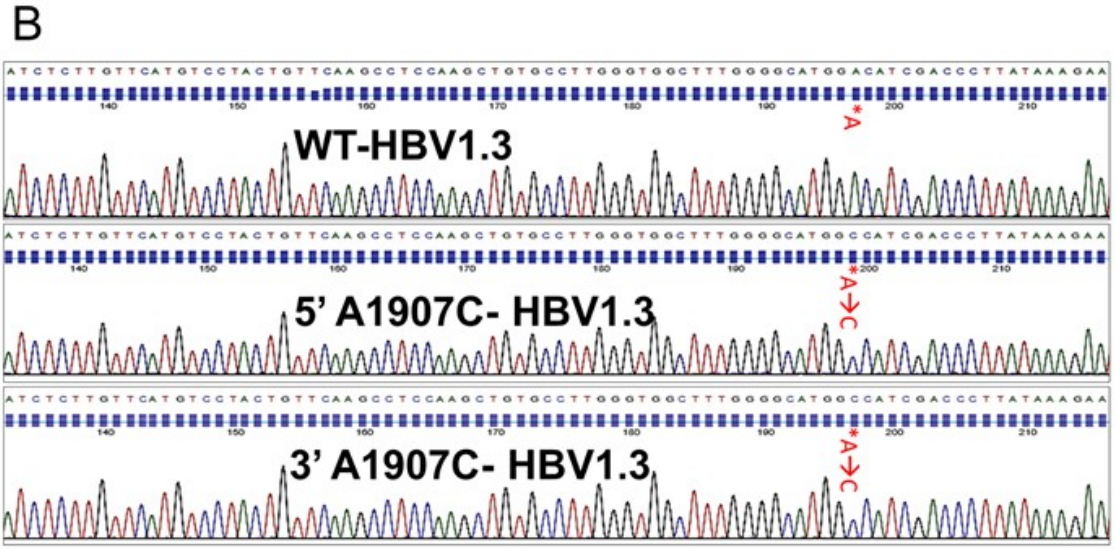
PreS2 mRNA
PreS1 mRNA
pgRNA
HBV Transcripts


B
WT-HBV1.3
5' A1907C- HBV1.3
3' A1907C- HBV1.3
A→C

SI References

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