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A hepatitis B virus transgenic mouse model with a conditional, recombinant, episomal genome

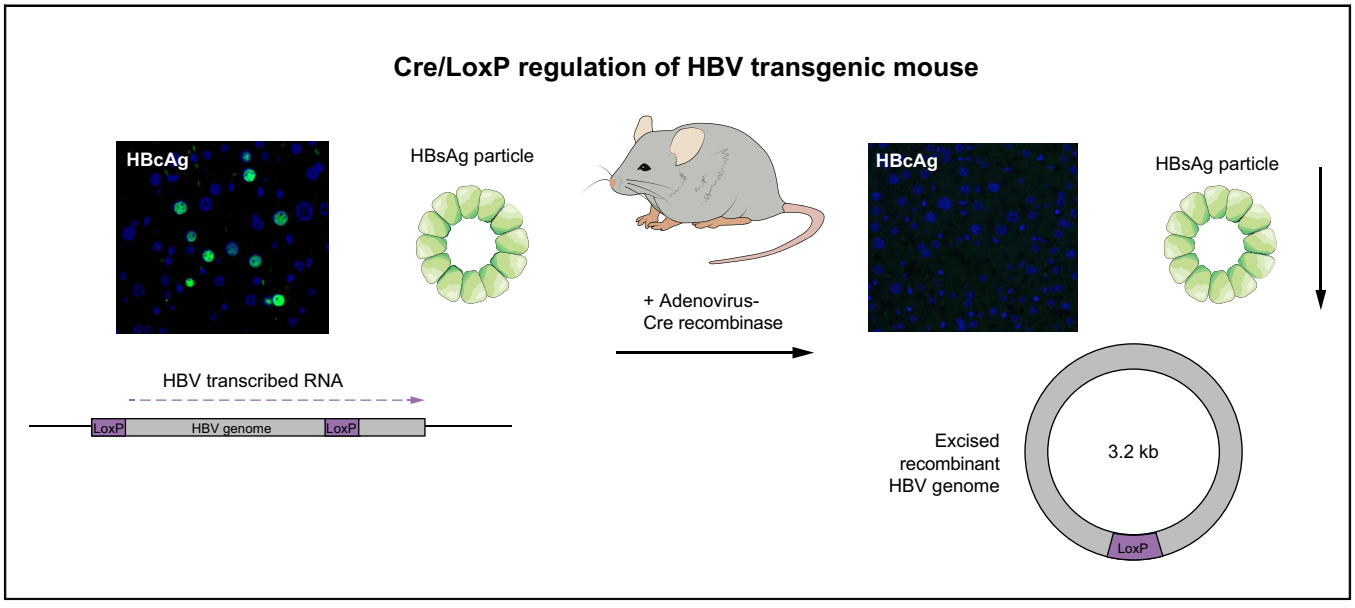
Authors

Robert L. Kruse, Mercedes Barzi, Xavier Legras, Francis P. Pankowicz, Nika Furey, Lan Liao, Janming Xu, Beatrice Bissig-Choisat, Betty L. Slagle, Karl-Dimiter Bissig

Correspondence

karldimiter.bissig@duke.edu (K.-D. Bissig).

Graphical abstract



Highlights

- Mouse models with the HBV genome integrated into mouse chromosomes are widely used to mimic chronic HBV infection.
- HBV integration prevents mice from being cured, so a novel transgenic mouse with HBV genome excision was developed.
- Cre/LoxP technology efficiently converted integrated HBV DNA into a recombinant circular genome similar to HBV cccDNA.
- Ad-Cre injection into our HBV transgenic mice eliminated HBV protein production and induced an immune response.

Lay summary

Hepatitis B virus (HBV) can only naturally infect humans and chimpanzees. Mouse models have been developed with the HBV genome integrated into mouse chromosomes, but this prevents mice from being cured. We developed a new transgenic mouse model that allows for HBV to be excised from mouse chromosomes to form a recombinant circular DNA molecule resembling the natural circular HBV genome. HBV expression could be reduced in these mice, enabling curative therapies to be tested in this new mouse model.

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A hepatitis B virus transgenic mouse model with a conditional, recombinant, episomal genome

Robert L. Kruse,[1,2,3,4] Mercedes Barzi,[5,6] Xavier Legras,[5,6] Francis P. Pankowicz,[1,2,7] Nika Furey,[1,2,5,6] Lan Liao,[7] Janming Xu,[7] Beatrice Bissig-Choisat,[1,2,5,6] Betty L. Slagle,[8] Karl-Dimiter Bissig[1,2,3,5,6,7,9,10,*]

[1]*Center for Cell and Gene Therapy, Texas Children's Hospital, Houston Methodist Hospital, Baylor College of Medicine, Houston, TX, USA;* [2]*Center for Stem Cells and Regenerative Medicine, Baylor College of Medicine, Houston, TX, USA;* [3]*Translational Biology and Molecular Medicine Program, Baylor College of Medicine, Houston, TX, USA;* [4]*Medical Scientist Training Program, Baylor College of Medicine, Houston, TX, USA;* [5]*Department of Pediatrics, Division of Medical Genetics, Duke University, Durham, NC, USA;* [6]*Y.T. and Alice Chen Pediatric Genetics and Genomics Research Center, Duke University, Durham, NC, USA;* [7]*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA;* [8]*Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, USA;* [9]*Duke Center for Virology, Duke University, Durham, NC, USA;* [10]*Duke Cancer Institute, Duke University, Durham, NC, USA*

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Background & Aims: Development of new and more effective therapies against hepatitis B virus (HBV) is limited by the lack of suitable small animal models. The HBV transgenic mouse model containing an integrated overlength 1.3-mer construct has yielded crucial insights, but this model unfortunately lacks covalently closed circular DNA (cccDNA), the episomal HBV transcriptional template, and cannot be cured given that HBV is integrated in every cell.
Methods: To solve these 2 problems, we generated a novel transgenic mouse (HBV1.1X), which generates an excisable circular HBV genome using Cre/LoxP technology. This model possesses a HBV1.1-mer cassette knocked into the *ROSA26* locus and is designed for stable expression of viral proteins from birth, like the current HBV transgenic mouse model, before genomic excision with the introduction of Cre recombinase.
Results: We demonstrated induction of recombinant cccDNA (rcccDNA) formation via viral or transgenic Cre expression in HBV1.1X mice, and the ability to regulate HBsAg and HBc expression with Cre in mice. Tamoxifen-inducible Cre could markedly downregulate baseline HBsAg levels from the integrated HBV genome. To demonstrate clearance of HBV from HBV1.1X mice, we administered adenovirus expressing Cre, which permanently and significantly reduced HBsAg and core antigen levels in the murine liver via rcccDNA excision and a subsequent immune response.
Conclusions: The HBV1.1X model is the first Cre-regulatable HBV transgenic mouse model and should be of value to mimic chronic HBV infection, with neonatal expression and tolerance of HBV antigens, and on-demand modulation of HBV expression.
Lay summary: Hepatitis B virus (HBV) can only naturally infect humans and chimpanzees. Mouse models have been developed with the HBV genome integrated into mouse chromosomes, but this prevents mice from being cured. We developed a new transgenic mouse model that allows for HBV to be excised from mouse chromosomes to form a recombinant circular DNA molecule resembling the natural circular HBV genome. HBV expression could be reduced in these mice, enabling curative therapies to be tested in this new mouse model.

Introduction

Hepatitis B virus (HBV) is a global pandemic with 248 million people estimated to be HBsAg-positive worldwide in 2010.[1] As a hepadnavirus, HBV forms a covalently closed circular DNA (cccDNA) transcriptional template inside the nuclei of host liver cells.[2] The episomal HBV cccDNA is exceptionally stable in the liver of patients after establishment in hepatocytes.[3] There is no curative therapy available against HBV and proposed therapeutic goals for HBV elimination revolve around silencing or degrading cccDNA. Unfortunately, there are currently no good models to study cccDNA.

HBV only replicates efficiently in humans and chimpanzees, which significantly limits research in this field. One reason for this limitation is hypothesised to be a block at the level of viral entry, restricting infection in other host species.[4] The human sodium taurocholate cotransporting polypeptide has been discovered as the receptor for HBV, but the introduction of the gene into mouse liver does not render the animals permissive for HBV infection.[5] One key issue is that mouse models are unable to form cccDNA.[6] Human liver chimeric mouse models are able to model entry and cccDNA formation, but the mice are immunodeficient and thus unable to model therapies that depend on the adaptive immune response against the virus.[7]

Keywords: Hepatitis B virus; Transgenic mouse; Cre/LoxP; cccDNA.
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* Corresponding author. Address: Department of Pediatrics, Division of Medical Genetics, Duke University, 905 South LaSalle St., Durham, NC, 27708, USA.
E-mail address: karldimiter.bissig@duke.edu (K.-D. Bissig).

To study HBV in convenient immunocompetent mouse models, HBV overlength genomes have been artificially introduced into murine liver, after which viral protein expression, DNA replication, and virion production occur. Delivery strategies have included adenovirus (Ad),[8] adeno-associated virus (AAV),[9] or hydrodynamic plasmid transfection.[10] In addition, a transgenic HBV mouse model has been created, possessing a linear 1.3 overlength genome integrated into host chromosomes.[11] The HBV transgenic mouse model has been a great advancement to the field, allowing study of the interaction of the virus with the immune system. The biology of this transgenic mouse resembles neonatal transmission, the predominant route of HBV infection, as HBV genes express from birth facilitating an immunotolerant state in the animal. Attempts to use vaccines to break immunotolerance in the mouse and trigger an HBV immune response have had mixed success.[12,13] However, as the HBV genome is integrated into every cell, only suppression of viral gene expression and not cure is possible.[14]

Given that the elimination of cccDNA cannot be achieved by therapies at present, there is a need to develop mouse models with cccDNA that resemble the immunotolerant state of HBV during chronic infection. To improve on the current HBV transgenic mouse model, we propose the creation of a mouse model with a recombinant cccDNA-like (rcccDNA) molecule from genomically integrated HBV using a recombinase system to overcome current experimental hurdles. A tolerated site in the HBV genome could be used to insert a LoxP sequence, recognisable by Cre recombinase. Herein, we describe the generation of this transgenic mouse model with inducible rcccDNA formation, using Cre/LoxP technology. The mouse resembles the current HBV transgenic mouse model in regard to HBV antigen tolerance during chronic infection, but with the advantage to excise the integrated HBV DNA. This ability to regulate the HBV genome and expression levels on demand should facilitate the establishment of chronic HBV mouse models.

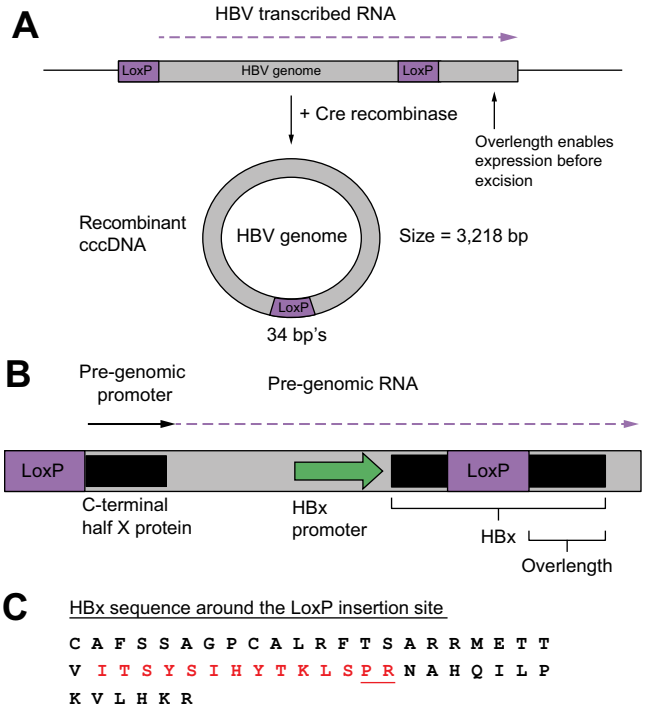

Fig. 1. A novel transgenic HBV mouse was designed with LoxP sites to facilitate excision of HBV genome. (A) A LoxP-flanked HBV genome was cloned with downstream HBV-overlength sequences maintaining open reading frames and supplying native polyadenylation for HBV protein expression. The LoxP-HBV cassette is excised by Cre recombinase to form a recombinant HBV cccDNA molecule. (B) The HBV1.1X cassette allows for complete expression of the pgRNA transcript and complete HBx protein to be expressed during integration. (C) The amino-acid sequence for the inserted LoxP site (red) and SacII site (underlined) at the HBx/overlength junction is depicted. cccDNA, covalently closed circular DNA; HBx, HBV X-protein; pgRNA, pregenomic RNA.

Materials and methods

HBV1.1X DNA construction

The plasmid, pSP65-ayw1.3, encoding a genotype D HBV genome (GenBank: V01460.1) (gift of Dr. Stefan Wieland, University of Basel) was used as a template to make the HBV1.1X sequence. Two fragments of HBV genome were generated by PCR, the external primers adding LoxP sites and the internal primers using a restriction site located within the HBV. A third fragment was an HBV sequence, bp 1156–1562 in GenBank: V01460.1, containing the c-terminal fragment of the HBx open reading frame (ORF) and an HBV sequence shortly past the polyadenylation site in the genome, while adding SacII to NheI sites to both ends to facilitate cloning. This c-terminal fragment has the same pregenomic (pg)RNA 3′-end as the wild-type HBV genome and the HBV 1.3-mer plasmids. See Table S1 for complete primer listing to generate these fragments.

General scheme of ligation to form pHBV1.1X: 5′-SmaI – LoxP – HBV sequence - Internal HBV restriction site (XhoI) + 5′-Internal HBV restriction site (XhoI) – HBV sequence – LoxP – SacII-3′ + 5′-SacII – HBV overlength sequence - NheI-3′ into a cloning site-modified pUC57 plasmid. Sequence information for the location of the LoxP insertion in the HBV genome at the amino acid level is provided in Fig. 1C. The vector maintains the entire HBx ORF and has a downstream polyadenylation sequence for production of pgRNA, similar to other overlength HBV vectors.

This transgene cassette was then cloned into a pROSA26-FRT-Neo plasmid containing homology arms to the murine *ROSA26* locus, which was derived from pROSA26-1 (Gift from Philippe Soriano, Addgene plasmid #21714).[15] The plasmid already contained an FRT-flanked PGK-Neo sequence that would be used for selection. The orientation was antisense to the *ROSA26* promoter (see Fig. S1) to avoid confounding effects of the ubiquitous promoter. Using SmaI and PacI sites for cloning, the final plasmid was named pROSA26-HBV1.1X-Neo.

All plasmid cloning was verified by sequencing (Lone Star Labs, Houston, TX, USA). To check for plasmid size where indicated, restriction digestion using restriction enzymes (New England BioLabs, Ipswich, MA, USA) run on a 1% agarose gel was used.

Creation of HBV transgenic mouse

pROSA26-HBV1.1X-Neo was then electroporated into murine embryonic stem cells (JM8A3 mouse C57/bl cells)[16] by the Mouse Embryonic Stem Cell Core (Baylor College of Medicine, Houston, TX, USA), who selected with G418 for positive clones containing the PGK-Neomycin gene. Southern blot analysis was used to verify successful integration into the murine *ROSA26* locus, using DIG non-isotopic detection system (Roche Applied Biosciences Penzberg, Germany) following the manufacturer's instructions.

Genomic DNA from positive-selected embryonic stem cell clones was digested with NsiI and the proper integration was analysed using a probe that binds upstream of the 3′ homology region of the pROSA26-HBV1.1X-Neo cassette. Digestion with NsiI releases a 19.3-kb band in the negative clones. As the HBV1.1X-Neo cassette contains an NsiI site within the genome, enzyme digestion generated an additional band of 15.4 kb only in the positive clones. A probe against the Neomycin cassette was used to verify integration (Fig. S2). We also validated the correct integration on both sides by PCR using a primer inside the integrated region and one in the mouse chromosome, outside the homology region. Sequencing results of the PCR product show a precise integration of the targeting vector into the ROSA26 murine locus (see Table S1 for primers utilised), as expected.

Among positive clones, mouse embryonic stem cell clones properly integrated were injected into blastocysts and implanted into female C57BL/6 mice, and chimera offspring selected. Chimera breeding with female albino mice were used to uncover black mice with germline transmission of the HBV1.1X-Neo transgene. Genotyping was performed by Transnetyx (Cordova, TN, USA). Breeding schemes outlined in Fig. S1 were then used to generate hetero- and homozygous mice, respectively. To delete the PGK-Neomycin cassette, we crossed the HBV1.1X-Neo line with the FLPeR strain (Jackson Laboratory, Bar Harbor, ME, USA), which expresses Flp recombinase under the control of the *ROSA26* promoter.[17]

Animal experiments

All animal experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. The *ROSA26*-mTmG mouse was obtained from Jackson Laboratory.[18] Alb-Cre mice were originally described in the enclosed reference (gift of Deborah Johnson, Baylor College of Medicine).[19] *ROSA26*-CreERT2 mice were obtained from Jackson Laboratory.[20]

Hydrodynamic tail-vein injection was used to introduce plasmid DNA in murine livers where indicated.[21] Briefly, plasmid DNA was diluted into normal saline 10% fluid body volume and then injected through the lateral tail vein over 4–6 s. The pCMV-NLS-Cre plasmid for these experiments was derived from pHR-CMV-nlsCRE (Gift from Didier Trono, Addgene plasmid #12265[22]). For experiments involving the injection of HBV1.1X with viral vectors, Ad-GFP and Ad-Cre were obtained from the Vector Development Lab (Baylor College of Medicine). Adenovirus (10^9 plaque-forming units) diluted into 200 μl PBS was injected into the tail vein of all mice. For the tamoxifen induction of *ROSA26*-CreERT2 mice, tamoxifen induction over 5 days was performed as previously described.[20]

For all blood draws, retro-orbital bleeding was used to obtain blood, after which blood was spun down for 30 min at 2.3×g and serum collected. After sacrifice and dissection, fragments of murine liver tissue were snap-frozen in liquid nitrogen and maintained at that temperature until analysis. DNA was extracted from tissues using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and tested for Cre excision products.

Primers to test for recombination over the LoxP junction in the DNA circle, that otherwise would not yield PCR products from the template cassette itself. Primers were also designed (see Table S1 for sequences) against the excised *ROSA26* genome locus to detect recombination events (see Fig. S3). PCR was designed to amplify only bands up to 1 kb according to the manufacturer's protocol (Phusion High-Fidelity PCR kit, New England Biolabs, Ipswich, MA, USA).

Southern blot analysis for HBV recombinant cccDNA

Southern blot to detect rcccDNA formation was performed on liver tissue from homozygous HBV1.1X male mice injected with Ad5-CRE. Extracted DNA was analysed by Southern blot using the DIG non-isotopic detection system (Roche Applied Biosciences) following the manufacturer's instructions. HBV1.1X non-injected males and a wild-type mouse were used as controls. Briefly, a probe was designed against the HBV genome and *Mfe*I was used to digest the genome at a single point, illustrating differences in migration between supercoiled and digested forms of rcccDNA.

Histology

Sections of paraffin-embedded liver were de-waxed and rehydrated, and rabbit anti-HBc primary antibody (Dako/Agilent, Santa Clara, CA, USA) applied. After washing the primary antibody with PBS 1×, slides were incubated at room temperature for 1 h with Alexa-Fluor secondary antibody (Molecular Probes, Eugene, OR, USA). Vectashield plus DAPI (Vector Labs, Burlingame, CA, USA) was used for slide mounting.

Serum analysis

Serum HBsAg levels (units as ng/ml) were quantified using commercially available ELISA reagents (International Immuno Diagnostics, Foster City, CA, USA) and HBsAg standards (Alpha Diagnostic International, San Antonio, TX, USA). Serum HBV DNA levels were determined by quantitative PCR as previously described.[23] Serum HBsAg IgG antibody levels were quantified by ELISA according to the manufacturer's instructions (Alpha Diagnostic International). HBsAg IgG antibody levels were reported as mIU/ml.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). Data measurements are presented as mean ± standard error of mean (SEM). Mean differences were tested using appropriate tests including unpaired, parametric, 2-tailed *t* tests. Significance level used was $p < 0.05$. Preliminary data determined effect size in all experiments, which guided appropriate power analysis for the sample size of each group.

Results

Designing a new HBV transgenic mouse with inducible episomal rcccDNA

Our goal was to design a mouse model wherein HBV proteins could be expressed from birth from an integrated genome, whereafter this integrated HBV genome could be excised to form rcccDNA (Fig. 1A). The excised rcccDNA would not amplify because of a lack of cccDNA formation in mice,[6] leading to a slow loss with hepatocyte mitosis over time. To accomplish this goal, we sought to mimic the currently used HBV overlength 1.3-mer genome, designed to facilitate production of all viral proteins. The overlength region duplicates part the HBV X-protein (HBx) ORF, so we sought locations in the HBx protein that could tolerate manipulation. We chose a peptide insertion strategy to avoid deleting important protein domains essential for the function of HBx, with the idea that the extra 12 amino acids

found in the LoxP site would just form an extra loop or linker in a protein and not cause interference. The region in HBx between the 2 protein motifs necessary for transcriptional trans-activation,[24] but after the polymerase coding sequence ends and before the beginning of the Enhancer II region,[25] was deemed a suitable target (Fig. 1B).

A PCR-mediated cloning strategy was used to insert a LoxP site into the HBx ORF (Fig. 1C). For HBV expression cassette in the mouse, we chose a 1.1-mer design affording expression of all viral proteins, similar to the current HBV transgenic mouse 1.3-mer, but with the benefit of leaving no residual full-length ORFs of HBV proteins after LoxP-excision, yielding clean elimination of viral proteins after Cre recombination. Only a portion of the c-terminal sequence of the HBx protein remains in the mouse genome, which should not be expressed as a result of a lack of start codon and promoter. The 1.1-mer overlength construct with LoxP insertion into the HBx gene was termed HBV1.1X. We tested the capability of rcccDNA generated from the LoxP insertion into HBx to mediate HBV replication and virus formation in a hydrodynamic tail vein injection model in a different study from our lab.[26] We found that rcccDNA could generate high HBV DNA levels in the serum that lasted for at least 2 months,[26] suggesting that all steps of HBV replication, including reverse transcription and virus packaging, were functional and that the chimeric HBx protein was active to prevent rcccDNA silencing.[27] Constructs of rcccDNA generated with null mutations in HBx generated no HBV core protein by comparison.[26] Thus, we proceeded forward with the LoxP insertion strategy into HBx in generating the transgenic mouse model.

We targeted the HBV1.1X construct to the *ROSA26* locus in mouse embryonic stem cells, chosen for its high-level expression and safe harbour locus in the genome. HBV1.1X was integrated in reverse orientation to the endogenous *ROSA26* promoter to guard against possible ubiquitous expression of HBV antigens (Fig. S1A), although previous studies have shown lack of read-through transcription and equal transcriptional potency in the ROSA26 locus regardless of orientation.[28] The cassette included a PGK-Neomycin sequence (combined sequence termed HBV1.1X-Neo) that facilitated selection in mouse embryonic stem cells (Fig. S1A). The initial HBV1.1X-Neo heterozygous or homozygous mice, however, did not have detectable HBsAg or HBc expression, both in the integrated state and after the introduction of Cre recombinase plasmid by hydrodynamic tail-vein injection (Fig. S4). As previous work reported silencing induced by the neomycin expression cassette,[29] we crossed some of our founders with a Flp recombinase strain[17] to remove the FRT-flanked PGK-Neomycin cassette (Fig. S1B).

HBV1.1X transgenic mice express HBV antigens and can form rcccDNA

The resulting HBV1.1X mice had detectable HBsAg in the serum. Male heterozygous mice expressed HBsAg levels at an average of 275 ± 73 ng/ml, whereas female mice expressed an average of 58 ± 13 ng/ml HBsAg (p = 0.026; Fig. 2A). In the male mice, detectable clusters of HBc-positive cells were found in their livers, amidst a majority of HBc-negative cells (Fig. 2B). HBc-positive cells were more frequently located in centrilobular regions near hepatic veins, similar to the location of cells

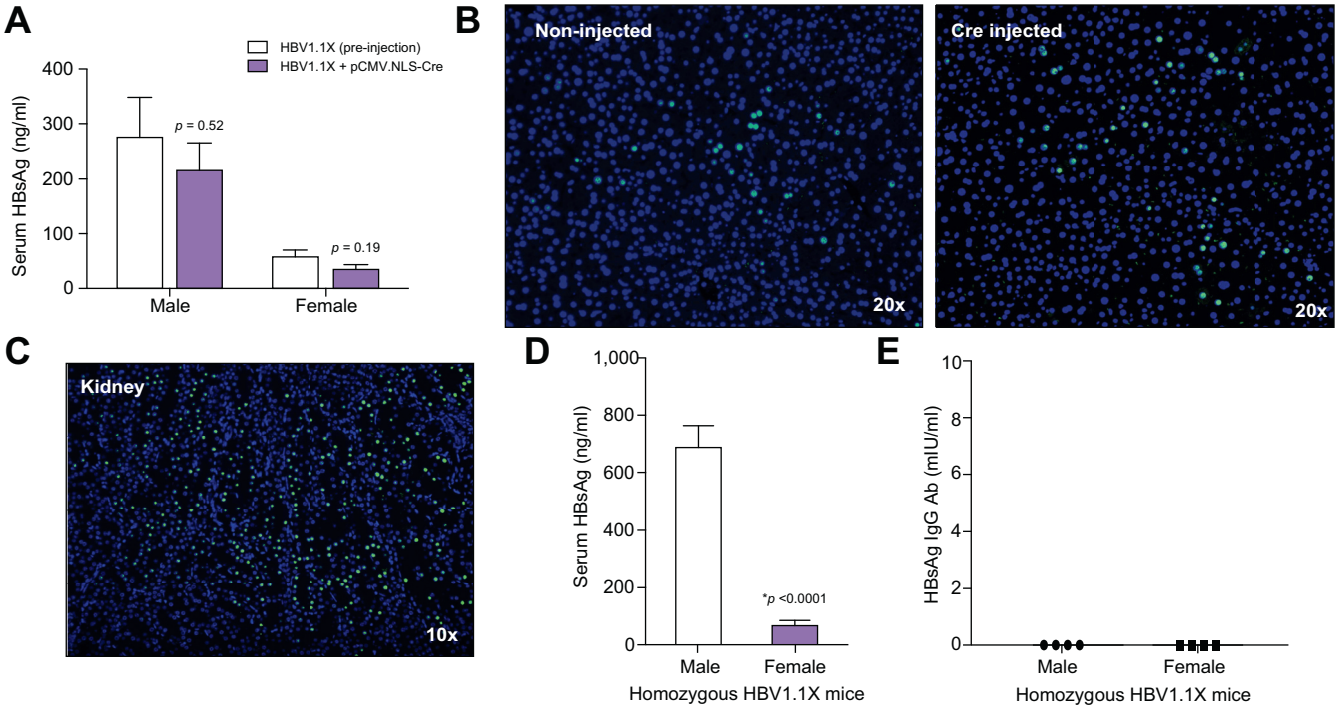

Fig. 2. HBV1.1X mice exhibit HBc expression in a small number of hepatocytes. (A) Hydrodynamic injection of pCMV-NLS Cre into heterozygous HBV1.1X mice, demonstrated no difference in HBsAg levels before and after Cre plasmid injection (n = 4). (B) Immunofluorescent staining in male mice showed a small percentage of HBc-positive hepatocytes, the frequency did not increase with Cre injection. (C) HBc expression could be detected in the renal tubule cells in the kidneys of mice, consistent with previous HBV transgenic mice. (D) Homozygous HBV1.1X mice exhibited high HBsAg expression levels at baseline (n = 4). (E) Male and female homozygous mice, aged 3 months, were assessed for the presence of HBsAg IgG antibodies, with both below background detection limit by ELISA assay. Bars represent mean and SEM; *significant, p <0.05 using parametric, 2-tailed t tests.

expressing cytoplasmic HBc and highly replicating DNA in HBV1.3 transgenic mice.[11] Interestingly, in the heterozygous female HBV1.1X mice, we could not observe any HBc-positive cells in the liver (data not shown). In both heterozygous male and female HBV1.1X mice, frequent HBc-positive cells were detected in the renal tubules, similar to the HBc staining described for the HBV1.3 transgenic strain (Fig. 2C). After generating homozygous HBV1.1X lines, we observed an increase in HBc-positive cells in the liver of male mice, whereas female homozygous mice now had detectable HBc-positive hepatocytes (Fig. S5). The HBsAg levels increased in male homozygous mice (688 ± 76 ng/ml; p <0.006), but not in female mice (67 ± 18 ng/ml; p = 0.70; Fig. 2D).

We next asked what would happen when Cre recombinase was injected by hydrodynamic tail-vein injection into the HBV1.1X mice, which results in plasmid delivery in 1–10% of hepatocytes.[30] Injecting 25 μg pCMV-NLS-Cre into HBV1.1X mice had no statistically significant change in HBsAg levels before and after Cre injection in male (216 ng/ml ± 49 ng/ml, p = 0.52) or female mice (35 ± 9 ng/ml p = 0.19; Fig. 2A). Staining for HBc-positive hepatocytes also showed no apparent differences between groups (Fig. 2B). By comparison, when 10 μg or 25 μg pCMV.NLS-Cre was injected into *ROSA26*-mTmG mice,[18] a mouse strain expressing membranous GFP upon Cre introduction, approximately 15–25% of mouse hepatocytes became positive for membranous GFP staining (Fig. S6). Given that a similar frequency of HBc-positive cells was not found in HBV1.1X mice after hydrodynamic tail-vein injection of Cre, this indicates that Cre was not activating HBc expression in these cells.

We also examined immunological and other viral features of the HBV1.1X strain. We were unable to detect serum HBV DNA by qPCR in heterozygous mice with or without Cre addition (limit of detection 10^4 copies/ml). This is similar to a previous report, which found the shorter 1.1-mer HBV construct in transgenic mice did not replicate HBV DNA to significant levels compared with the 1.3-mer overlength version.[11] We were unable to detect any anti-HBsAg IgG antibodies in either male or female HBV1.1X homozygous mice at 3 months of age, indicating tolerance to HBsAg similar to human HBV patients (Fig. 2E). Furthermore, at baseline, there was no pronounced inflammation of the liver as assessed by haematoxylin and eosin stain in non-infected mice (data not shown).

We performed a Southern blot to confirm proper rcccDNA formation. Using homozygous transgenic HBV1.1X mice injected with or without Ad-Cre, we harvested the livers 1 day post-injection and isolated DNA from liver tissue. On Southern blot, we could discriminate between the integrated transgene and rcccDNA forms by size (Fig. 3A). The faster migration on gel electrophoresis of non-digested *vs.* *Mfe*l-digested linear DNA is consistent with supercoiling of the uncut rcccDNA (Fig. 3B). One important difference is the rcccDNA band had an apparent migration of ~2.8 kb, whereas most reports show authentic HBV cccDNA migrating at 1.8–2.1 kb,[31,32] emphasising a potential difference in supercoiling and/or histone organisation between the 2 DNA molecules. Equally important, the Southern blot did not show any HBV DNA replicative intermediates, consistent with the absence of HBV DNA in the serum and emphasising the low-level transcription from the integrated genome.

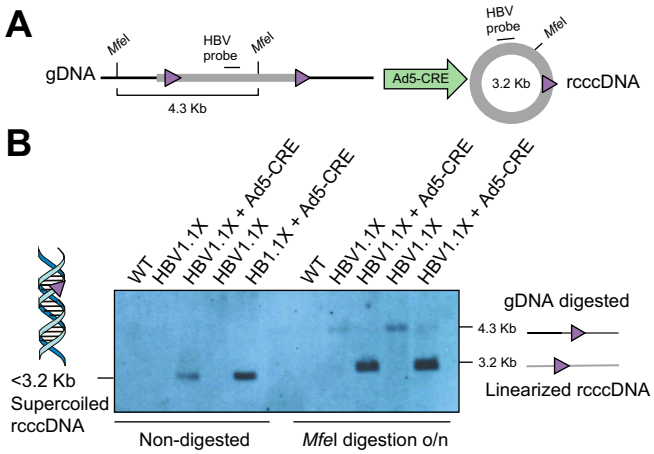

Fig. 3. Southern blot demonstrates rcccDNA formation from integrated HBV1.1X genome after Cre recombinase introduction. A representative diagram of HBV1.1X genome digestion for Southern blot is provided (A), where integrated HBV can be released with *Mfe*I digestion, or alternatively released after introduction of Ad-Cre. The probe is localised over the PreS region of the HBV genome. (B) Southern blot revealed a small band released with the introduction of Cre, while the control remains unstained. Upon *Mfe*I digestion, the integrated HBV DNA is released, while the rcccDNA form is now shifted upward owing to linearisation (~3.2 kb). Ad, adenovirus; rcccDNA, recombinant covalently closed circular DNA.

Transgenic Cre recombinase mouse tools eliminate HBsAg expression

We next asked if we could manipulate our HBV1.1X mice using 2 available transgenic mouse Cre/LoxP tools[33]: albumin-Cre (Alb-Cre) mice and tamoxifen-inducible Cre (Cre-ERT2) mice. We first obtained an Alb-Cre mouse strain[19] and crossed it with homozygous HBV1.1X male mice, yielding Alb-Cre/HBV1.1X mice. Alb-Cre would be expected to activate Cre-mediated excision specifically in the liver beginning shortly after birth, with the liver continuing to grow subsequently to adulthood, thereby diluting out episomal HBV DNA.[19] The liver grows approximately 9-fold from neonatal age to 3 weeks old.[34] We observed that male mice from the same litter that did not receive Alb-Cre allele continued expressing HBsAg (228 ± 53 ng/ml) and had detectable HBc expression in the liver (Fig. 4A and B). By comparison, Alb-Cre/HBV1.1X had no detectable HBsAg expression (<1 ng/ml, p = 0.013) at the time of weaning at 3 weeks old or at any time thereafter (Fig. 4A). Similarly, immunofluorescence revealed no detectable HBc expression (Fig. 4B). Female mice showed a similar pattern (72 ± 6 ng/ml *vs.* <1 ng/ml, p = 0.0002). The kidneys of the Alb-Cre/HBV1.1X mice continued expressing HBc in all mouse groups, confirming the specificity of the Alb-Cre effect to the liver (data not shown).

We next investigated the use of a tamoxifen-inducible Cre recombinase (CreERT2) as a method of excising the HBV genome on demand at any time point.[35] We obtained *ROSA26*-CreERT2 mice, which have ubiquitous CreERT2 expression and efficiently excises floxed sites from alleles after a short tamoxifen-induction course,[20] and proceeded to cross them with our HBV1.1X mice. We found that HBV1.1X/CreERT2 mice at 8–12 weeks expressed similar levels of HBsAg among the male (210 ± 33 ng/ml, p = 0.37) and female mice (95 ± 15 ng/ml, p = 0.15) compared with the heterozygous HBV1.1X mice previously generated (Fig. 4C). After a 5-day induction course with tamoxifen, we observed a dramatic decrease in HBsAg levels in both male (4.0 ± 1.8 ng/ml, p <0.0001) and female (2.5 ± 0.7 ng/ml, p <0.0001) mice, close to the detection limit (Fig. 4C). This reduction in HBsAg levels was not as a result of anti-HBsAg IgG development, as mice had no

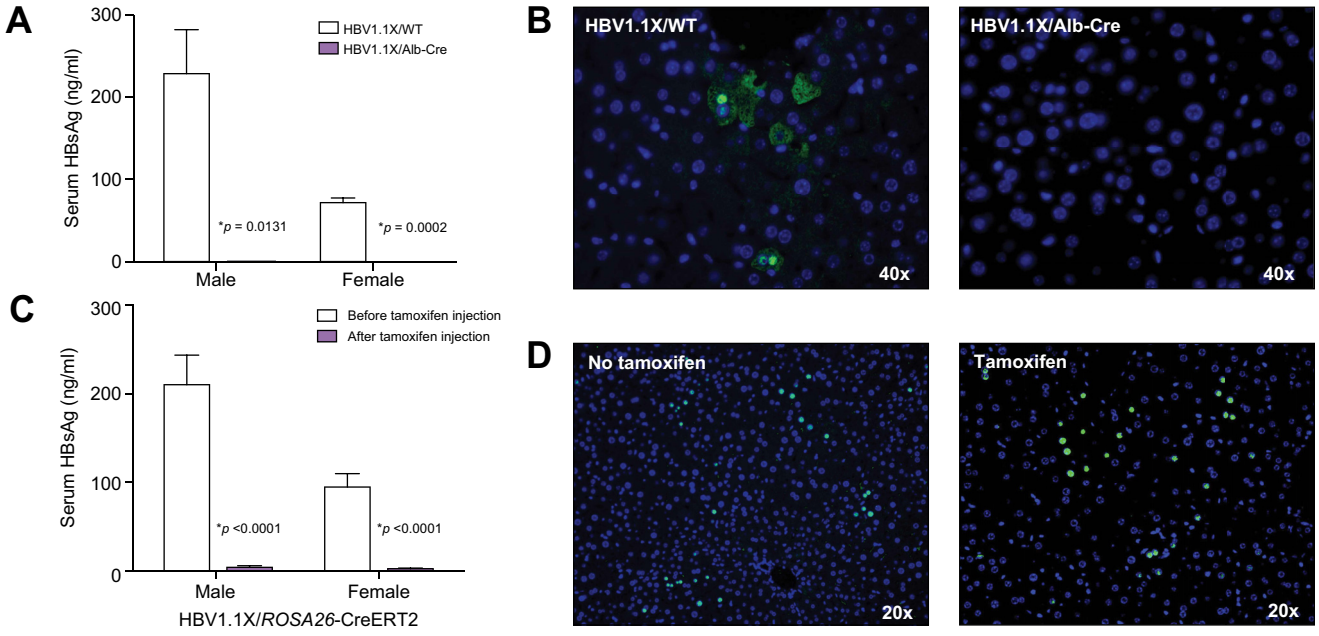

Fig. 4. HBsAg expression can be eliminated in HBV1.1X mice using transgenic Cre recombinase tools. HBV1.1X mice were crossed with Alb-Cre transgenic mice to induce liver-specific excision of the HBV genome. (A) Male HBV1.1X/Alb-Cre heterozygous for both alleles had undetectable serum HBsAg compared to control mice without Alb-Cre (n = 3). (B) Immunofluorescent staining for HBc protein in the livers of both mice showed less HBc staining of HBV1.1X/Alb-Cre mice. (C) HBV1.1X mice were crossed with *ROSA26*-CreERT2 mice to create *ROSA26*-CreERT2/HBV1.1X mice. Tamoxifen induction of CreERT2 resulted in complete loss of HBsAg expression 1 week after induction cycle ended (n = 8). (D) HBc staining in the liver of mice untreated and treated with tamoxifen revealed continued presence of HBc positive cells 3 months after tamoxifen induction period. Bars represent mean and SEM; *significant, p <0.05 using parametric, 2-tailed t tests. Alb, albumin.

detectable antibodies before or after tamoxifen injection (data not shown). Analysing the murine livers, we still observed a similar level of HBc staining in hepatocytes among tamoxifen-treated and non-treated mice (Fig. 4D). We confirmed excision of HBV DNA to form rcccDNA after tamoxifen treatment by PCR, demonstrating efficient excision (Fig. S5).

Viral Cre recombinase mice tools can be used to eliminate HBc expression

We next sought to demonstrate whether our HBV1.1X mice could effectively be purged of HBV proteins. We hypothesised that adenovirus expressing Cre could both mediate the Cre-mediated excision of HBV genome and induce inflammation to remove HBV rcccDNA episome molecules. Previous studies have reported that adenovirus is efficient at transducing hepatocytes,[36,37] but can activate a strong immune response.[38] Moreover, adenoviral transduction of HBV1.3 transgenic mice with reporter constructs can promote temporary clearance of HBV DNA replicative intermediates associated with T cell infiltration into the liver and IFN-gamma and TNF-alpha production.[39] We sought to mimic this same result and use bystander inflammation to permanently eliminate HBV expression via loss of the HBV episome, contrasting to the HBV1.3 transgenic mouse with integrated DNA.

We utilised 3 groups (Ad-Cre, Ad-GFP, and non-injected) injected into heterozygous HBV1.1X mice for this experiment. The Ad-GFP group would serve as the surrogate to Ad-LacZ to the previous study,[39] inducing inflammation but not excising the HBV genome. We observed that only the Ad-Cre mouse group had a marked decrease in HBsAg expression after Ad-Cre injection (17.3 ± 3.2 ng/ml), while the Ad-GFP (275 ± 52 ng/ml, p = 0.002) and non-injected (303 ± 69 ng/ml, p = 0.004) groups continued to maintain similar levels of HBsAg level expression (Fig. 5A). Longer-term rebounds in HBsAg levels were not observed (data not shown). Looking at HBc staining in the liver, we observed that the Ad-Cre treated mice lost almost all HBc-positive hepatocytes in the liver, while HBc-positive cells were found in Ad-GFP treated mice (Fig. 5B–D). We asked whether HBsAg and HBc clearance was attributable to the development of anti-HBsAg IgG antibody response, but were unable to detect any anti-HBsAg IgG antibodies in the Ad-Cre or the other groups, before or after injection (data not shown). Altogether, Ad-Cre decreased HBsAg levels like the transgenic Cre methods, but the inflammation from adenoviral treatment also removed HBc expression from rcccDNA excised mice, but not from control, unexcised mice.

Discussion

HBV1.3 transgenic mice have been a boon to the field of HBV research,[11] affording convenient models to study HBV immunology, replication, and therapeutics. However, they have 2 key limitations. First, despite their widespread use as a therapeutic testing tool, the mice cannot be cured of HBV. Only viral suppression with therapy or immunity can be studied as the HBV genome remains integrated in the chromosome of the mouse. Second, HBV1.3 transgenic mice lack cccDNA, which is the natural transcriptional template and thought to be the key barrier to elimination of the virus in human patients.

To address these limitations and generate a more accurate small animal model for HBV, we generated the HBV1.1X mouse.

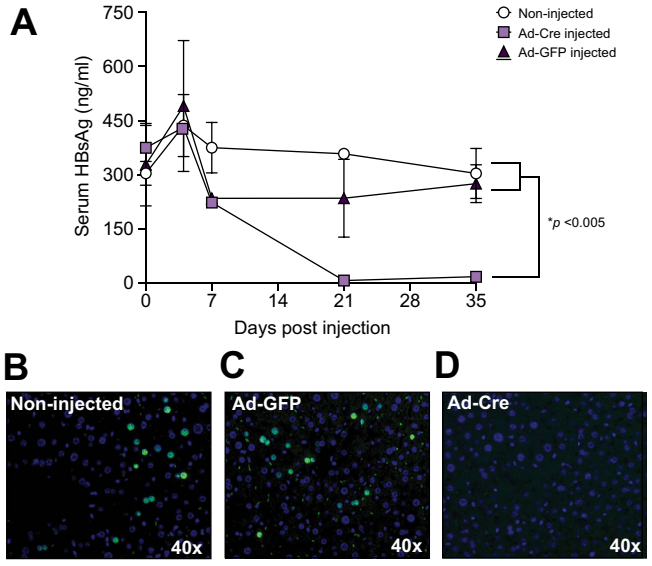

Fig. 5. Both HBsAg and HBc expression can be eliminated from the liver by administering Ad-CMV-Cre into mice. Heterozygous HBV1.1X male mice were obtained and injected with Ad-CMV-Cre (n = 4) or Ad-CMV-GFP (n = 3), or not injected (n = 3) with virus as a control. (A) Serum HBsAg levels were followed post-injection in the mice. All mice continued to express HBsAg over the first week post injection, but by Day 21, Ad-Cre mice had lost expression. Each point represents mean and SEM; *significant, p <0.05 using parametric, 2-tailed t tests. (B–D) Staining for HBc antigen by immunofluorescence at Day 34 post injection, observed the continued presence of HBc-positive cells in non-injected and Ad-CMV-GFP injected mice, while Ad-CMV-Cre mice lacked observed HBc staining, beyond rare hepatocytes (1 positive cell in a lobe). Ad, adenovirus.

This novel HBV transgenic mouse model exhibits tolerance to chronic antigen, but has the ability to remove HBV DNA from the genome using Cre recombinase, and the potential to ultimately clear rcccDNA from the murine liver permanently. We leveraged the Cre/LoxP system to artificially create rcccDNA within the mouse, given that mouse hepatocytes do not form this molecule.

While this study was in progress, other researchers developed similar but distinct approaches in leverage recombinase-mediated rcccDNA generation.[40–42] These studies used a different tolerated site[41,43] in the HBV genome for LoxP insertion, or alternatively inserted an artificial intron into the HBV genome[42] to facilitate viral replication. Improvements to hydrodynamic delivery of rcccDNA made by using replication-defective recombinant adenoviral vectors have been used to deliver recombinant HBV genome into Cre-expressing mice allowing rcccDNA to be generated.[40] However, these mouse models with rcccDNA are limited by variable progression to chronic infection because of antiviral immune responses. Chronic HBV persistence was often dependent on mouse strain and/or DNA dose injection, with conditions resulting in acute infection, representing a key limitation.[40–42] By contrast, our study did not require the injection of HBV DNA into mice, and the presence of HBV antigens at birth yielded an intrinsic HBV chronicity. Indeed, this is the first application of this rcccDNA strategy where the HBV DNA is already in the murine genome and expressed, thereby establishing a tolerant, chronic infection system. Our strategy bears some resemblance to a HepG2 cell line generated with integrated HBV genomes that can be excised with Cre recombinase,[44] but with the advantage of being in a living mouse strain.

After excision of rcccDNA in the HBV1.1X mouse, we observed that HBsAg levels became undetectable in the serum. As outlined in Fig. 6, we hypothesise that the transcriptionally active *ROSA26* locus may help drive HBsAg expression when the HBV1.1X cassette is integrated, but in the Cre-excised rcccDNA form, this additional transcriptional boost is lacking, leading to a dramatic drop in levels. We observed ~2–5% HBc-positive hepatocytes within mice that usually occurred in clusters of several hepatocytes, most often near the centrilobular region. However, outside these regions, there were large areas completely absent of expression, suggesting silenced HBV DNA in these cells, which was not activated by Cre recombinase introduction. By comparison, the HBV1.3 transgenic mouse expresses HBc in almost every cell, although at different intensities.[11] This difference of expression may be attributable to the lack of a second Enhancer I region in our HBV1.1X mice, which can drive higher expression of all HBV genes, while also potentially protecting from transgene silencing. By analogy, when the neomycin gene, which has been shown to silence expression in neighbouring transgenes,[29] was removed in our HBV1.1X mice, there was an increase HBV antigen expression. Our experience with seemingly silent HBV transgenes in a portion of hepatocytes was similar to others, who found that silencing by methylation of integrated HBV genes commonly occurs.[45] In this regard, it was likely helpful to integrate the transgene into the transcriptionally active *ROSA26* locus, which normally allows for stable and ubiquitous expression in mice, and in our case, consistently protected expression in a small percentage of hepatocytes. By contrast, there is wide variability of HBsAg expression

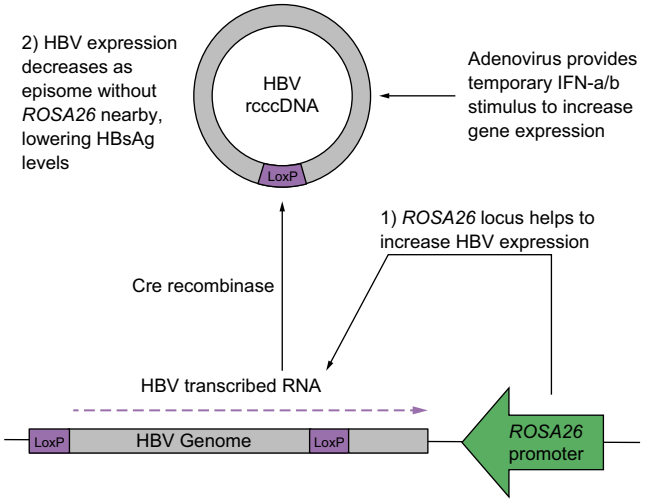

Fig. 6. Mechanism of gene expression regulation in HBV1.1X transgenic mice with Cre recombinase. A model is presented of how HBV gene expression may be regulated by Cre recombinase in HBV1.1X transgenic mice. The model predicts at baseline, the *ROSA26* locus helps drive expression of the HBV1.1X genome in a small fraction of hepatocytes, with the rest of the genomes transcriptionally silent. After Cre recombinase is introduced, the HBV genome is excised and converted into rcccDNA, which can continue expression, but expression levels are reduced without genomic context. The cellular environment may compensate by helping to activate gene expression of rcccDNA, such as seen after adenoviral introduction. Overall, HBsAg levels fall near the detection limit given the low level of HBV-expressing hepatocytes with 1 or 2 genomes expressing HBV antigens, much lower than the amount of copies delivered by hydrodynamic delivery of plasmids or viral vector approaches. rcccDNA, recombinant covalently closed circular DNA.

in the HBV1.3 transgenic mouse, ranging from 156 to 1169 ng/ml in male mice.[23] Moreover, this relatively low number of HBc-positive hepatocytes could also explain why we did not observe HBV DNA levels in the serum. This experience is similar to previous HBV transgenic mouse lines, which could not detect DNA replication with the shorter overlength (1.1- or 1.2-mer) versions.[11] Given the lack of HBV DNA in the serum and lack of HBV DNA replicative intermediates on Southern blot, the utility of this mouse model lies in regulating HBV protein expression and is not a good model for studying HBV replication or rcccDNA transcriptional regulation. Indeed, the apparent differences in supercoiling between rcccDNA and authentic cccDNA could also influence gene expression and suggest that any mouse chromosome-derived HBV rcccDNA presents a limitation for studying HBV cccDNA biology.

This HBV1.1X mouse model should be useful to establish immunocompetent HBV models of chronic HBV infection that are curable. Low-cost, convenient hydrodynamic tail-vein injection models using HBV plasmid typically establish variable, strain-dependent chronicity,[46] whereas Ad-HBV leads to rejection at high doses.[8] Only AAV-HBV appear to establish persistence at high efficiencies,[9,47] but its widespread use is limited by cost, and the maximum HBV titre achieved in AAV models is relatively low. By contrast, as our HBV1.1X mice will have low expression of all antigens from birth establishing neonatal tolerance, additional HBV DNA can then be injected into HBV1.1X mice, by any means, to increase viral levels with no concern for rejection. This will facilitate the consistent establishment of chronic HBV viraemia and antigenaemia in mice, particularly for HBV plasmid-based models.[10,46] The integrated DNA portion can be left in the genome, or completely excised using CreERT2 tools after injection to facilitate testing for a sterilising DNA-negative cure. In this regard, our model is useful in having low levels of HBV antigen expression, allowing one to easily distinguish between the contribution of the input levels of HBV DNA and the baseline HBV antigen expression from the mouse. Moreover, the rapid drop in HBsAg levels after excision by Cre-ERT2, for example, is a convenient marker for recombination, and will allow investigators to identify any HBsAg subsequently produced as coming from injected DNA, which could be from any genotype of HBV chosen.

In conclusion, this study has established a new HBV1.1X transgenic mouse model for HBV research, leveraging the artificial generation of rcccDNA via Cre recombinase from an integrated genome. As far as we know, this is also the first transgenic mouse model with Cre-regulatable HBV expression in mice. We demonstrated the ability to reduce HBV protein expression with Cre recombinase expression and dramatically reduce HBV antigens and HBV DNA from the mouse. Ultimately, we believe the versatility of these tools will benefit the field in more easily establishing chronic HBV infection models and studying questions of basic viral biology and immune clearance.

Abbreviations

AAV, adeno-associated virus; Ad, adenovirus; cccDNA, covalently closed circular DNA; HBx, HBV X-protein; ORF, open reading frame; PFUs, plaque-forming units; pgRNA, pregenomic RNA; rcccDNA, recombinant cccDNA.

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Conflicts of interest

The authors declare no conflicts of interest.
Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Designed experiments: RLK, BLS, K-DB. Immunostaining, transgenic targeting design, and Southern blotting: MB. ELISA assays, DNA extraction, and PCR: XL. Mouse injections: RLK, FPP, BB-C. Harvested tissue: RLK, MB, BB-C. Generated the transgenic mouse: LL, JX. Colony management: RLK, BB-C, NF. Read and approved the final version of the manuscript: all authors

Data availability

The data that support the findings of this study are available from the corresponding author, K.-D.B., upon reasonable request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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