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Hepatitis B virus enhancer 1 activates preS1 and preS2 promoters of integrated HBV DNA impairing HBsAg secretion

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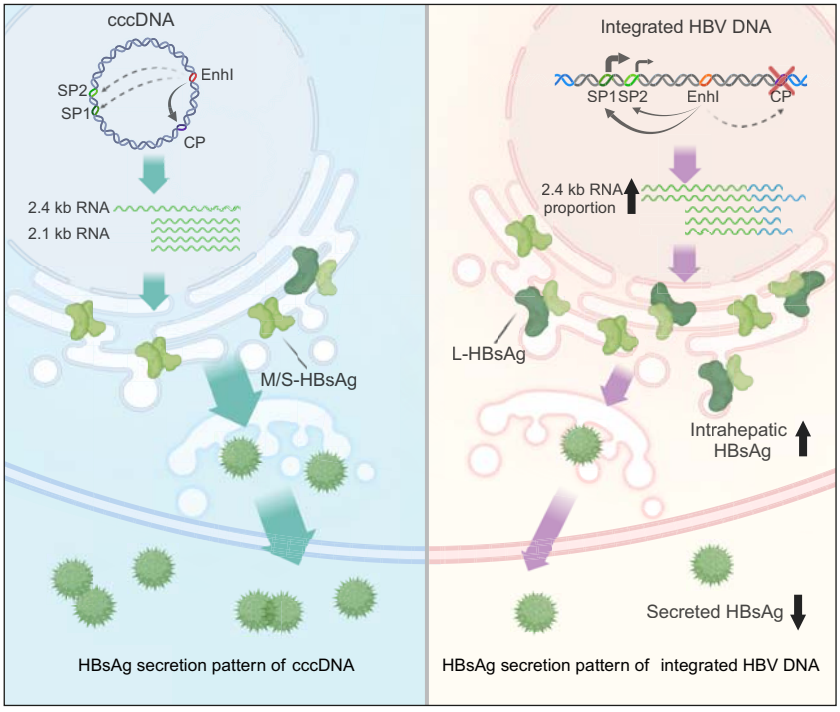
Graphical abstract



Highlights

- The secretion efficiency of HBsAg is lower in HBeAg-negative patients compared with HBeAg-positive patients.
- A high proportion of L-HBsAg inhibits the secretion of HBsAg derived from integrated HBV DNA.
- The functional deficiency of core promoter in integrated HBV DNA leads to uneven activation of preS1 and preS2 promoters by enhancer 1.

Impact and implications

Integrated hepatitis B virus (HBV) DNA can serve as an important reservoir for HBV surface antigen (HBsAg) expression, and this limits the achievement of a functional cure. This study revealed that secretion efficiency is lower for HBsAg derived from integrated HBV DNA than HBsAg derived from covalently closed circular DNA, as determined by the unique sequence features of integrated HBV DNA. This study can broaden our understanding of the role of HBV integration and shed new light on antiviral strategies to facilitate a functional cure. We believe our results are of great general interest to a broad audience, including patients and patient organisations, the medical community, academia, the life science industry and the public.

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Hepatitis B virus enhancer 1 activates preS1 and preS2 promoters of integrated HBV DNA impairing HBsAg secretion

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Background & Aims: The expression of HBsAg from integrated HBV DNA limits the achievement of functional cure for chronic hepatitis B. Thus, characterising the unique expression and secretion of HBsAg derived from integrated HBV DNA is of clinical significance.

Methods: A total of 563 treatment-naive patients and 62 functionally cured patients were enrolled, and HBsAg and HBcAg immunohistochemistry of their liver biopsy tissues was conducted followed by semi-quantitative analysis. Then, based on stratified analysis of HBeAg-positive and -negative patients, long-read RNA sequencing analysis, as well as an *in vitro* HBV integration model, we explored the HBsAg secretion characteristics of integrated HBV DNA and underlying mechanisms.

Results: In contrast to the significantly lower serum HBsAg levels, no significant decrease of intrahepatic HBsAg protein was observed in HBeAg-negative patients, as compared with HBeAg-positive patients. The results of long-read RNA sequencing of liver tissues from patients with chronic HBV infection and *in vitro* studies using integrated HBV DNA mimicking dslDNA plasmid revealed that, the lower HBsAg secretion efficiency seen in HBeAg-negative patients might be attributed to an increased proportion of preS1 mRNA derived from integrated HBV DNA instead of covalently closed circular DNA. The latter resulted in an increased L-HBsAg proportion and impaired HBsAg secretion. Enhancer 1 (EnhI) in integrated HBV DNA could retarget preS1 (SP1) and preS2 (SP2) promoters to disrupt their transcriptional activity balance.

Conclusions: The secretion of HBsAg originating from integrated HBV DNA was impaired. Mechanistically, functional deficiency of core promoter leads to retargeting of EnhI and thus uneven activation of the SP1 over the SP2 promoter, resulting in an increase in the proportion of L-HBsAg.

Introduction

Chronic hepatitis B virus (HBV) infection affects approximately 296 million people worldwide and is the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[1] During HBV replication, there are three surface proteins encoded within the single preS/S open reading frame (ORF) by alternate use of three in-frame start codons, collectively referred to as hepatitis B surface antigen (HBsAg). Small (S) HBsAg only contains S domain, middle (M) HBsAg is extended by preS2 domain. Large (L) HBsAg is extended by one more preS1 domain on M-HBsAg. HBsAg plays an important role in viral life cycle and modulation of host immune responses. Among them, S-HBsAg and L-HBsAg are essential for assembly and infectivity of virions, whereas M-HBsAg is dispensable for the production of infectious virions.[2]

In clinical practice, loss of HBsAg is the most important indicator of functional cure for chronic hepatitis B (CHB) and is considered the optimal endpoint of antiviral therapy.[3] However, it is difficult to achieve HBsAg loss in patients with CHB via the use of currently available nucleos(t)ide analogues (NAs) and/or pegylated-interferon-α (Peg-IFNα).[4] As the precursor for integration, double-stranded linear DNA (dslDNA) can integrate into the host genome to serve as another source of HBsAg in patients with CHB apart from covalently closed circular DNA (cccDNA).[5] Now it is widely accepted that clearance of integrated HBV DNA is important for achieving functional cure. However, expression and secretion patterns of HBsAg derived from integrated HBV DNA and underlying mechanisms remain unclear.

A decrease in proportion of L-HBsAg and M-HBsAg has been reported during treatment with NAs and Peg-IFNα in

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HBeAg-positive patients with CHB with HBsAg loss.[6] A recent retrospective cohort study also revealed that serum HBsAg composition exhibits special patterns at different stages of HBV infection.[7] Considering potentially different contributions of integrated HBV DNA and cccDNA to serum HBsAg during disease progression of chronic HBV infection,[8,9] the variable HBsAg composition across different stages suggests that the expression and secretion patterns of HBsAg might be different between integrated HBV DNA and cccDNA.

The transcription of cccDNA is regulated by two enhancers: enhancer 1 (EnhI) and enhancer 2 (EnhII). EnhII has strong effect on transcription of core promoter (CP), as well as preS1 (SP1) and preS2 (SP2) promoters, whereas EnhI has a modest effect on SP1 and SP2 promoters.[10] Unfortunately, insights into how SPs promoters maintain transcriptional activity to support HBsAg expression from integrated HBV DNA are lacking. Theoretically, the selectivity of enhancers to promoters is not constant, enhancers in mammalian cells can be released to activate alternative promoters in the case of functional loss of their partner promoters.[11] In addition, a recent study reported that EnhI in integrated HBV DNA could selectively regulate a host promoter.[12] Whether EnhI is involved in activating SPs promoters in integrated HBV DNA and thus shapes unique HBsAg expression and secretion characteristics remain to be determined.

Here, we comprehensively investigated expression and secretion patterns of HBsAg derived from integrated HBV DNA using retrospective cohorts and *in vitro* HBV-integration models. Our findings demonstrated that the secretion efficiency was lower for HBsAg derived from integrated HBV DNA than cccDNA. Mechanistically, EnhI could retarget and unevenly activate SP1 and SP2 promoters in integrated HBV DNA. Consequently, unbalanced L-HBsAg proportion shaped the unique HBsAg secretion pattern. These findings might provide clues to facilitate the achievement of functional cure for CHB.

Patients and methods

Information regarding patients and methods is shown in the Supplementary material.

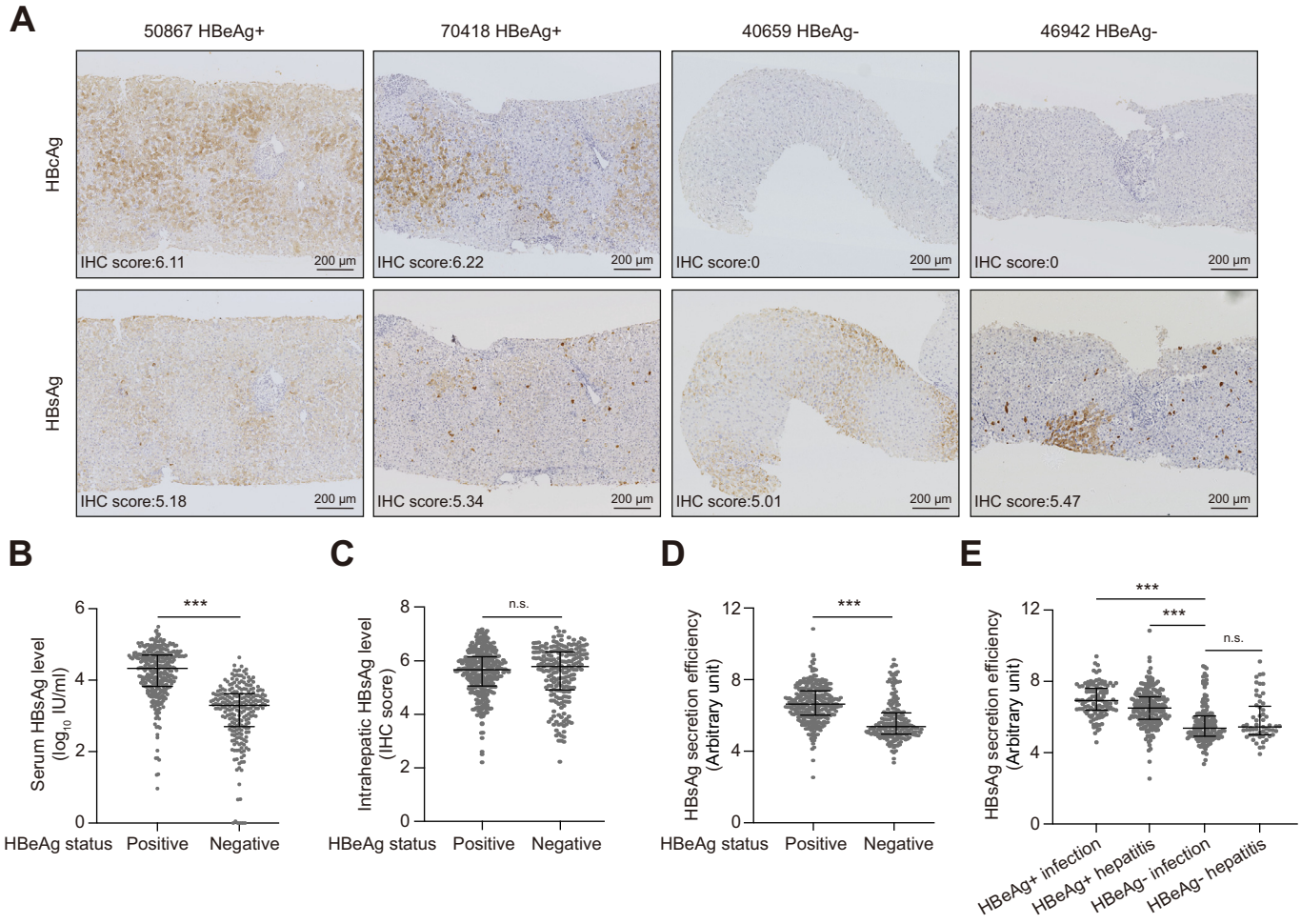

Fig. 1. The secretion efficiency of HBsAg was lower in HBeAg-negative patients than HBeAg-positive patients. (A) Representative IHC images of liver biopsy samples stained with anti-HBc and anti-HBs antibodies. The corresponding IHC scores were determined by average optical density, as detailed in the Supplementary Methods. (B and C) Levels of serum HBsAg (B) and intrahepatic HBsAg (C) were compared between HBeAg-positive and HBeAg-negative patients. (D and E) HBsAg secretion efficiency was compared between HBeAg-positive and HBeAg-negative patients (D), and in patients with different clinical phases (E). The HBsAg secretion efficiency was calculated as detailed in the Supplementary Methods. (Data are presented as median ± interquartile range. Two-tailed Mann–Whitney *U* test, ***p <0.001, NS, not significant). IHC, immunohistochemistry.

Table 1. Clinical features of 563 treatment-naive patients.

HBeAg status	HBeAg-positive (N = 329)		HBeAg-negative (N = 234)		*p* value*
Phases†	Chronic infection (N = 118)	Chronic hepatitis (N = 211)	Chronic infection (N = 174)	Chronic hepatitis (N = 60)	–
Sex (male/female)‡	72/46	130/81	94/80	39/21	0.277
Age (years)‡	31 (25, 37)	30 (26, 37)	41 (35, 48)	36 (30, 44.25)	<0.001
ALT (U/L)‡	34.5 (23.25, 53)	101.5 (43.25, 298.75)	21.5 (16, 33.75)	58.5 (29, 140.5)	<0.001
AST (U/L)‡	27 (21.25, 33.75)	65.85 (34, 147.25)	23 (19, 30)	42 (26, 82.25)	<0.001
GGT (U/L)‡	17 (13, 23)	31 (18, 60)	17 (13, 25)	27 (17, 47.5)	<0.001
ChE (U/L)‡	7051.5 (6226.25, 8552)	6473 (5611.25, 7341.75)	7376 (6490, 8767)	6707.5 (5744.25, 7787.75)	<0.001
PLT (10^9/L)‡	206 (176.25, 238.75)	189.5 (158.75, 218)	186.5 (153.5, 222)	177 (146.5, 205.25)	0.012
Serum HBV DNA (log_{10} IU/ml)‡	8.00 (7.69, 8.44)	7.87 (6.85, 8.24)	3.13 (2.40, 4.02)	4.44 (3.46, 6.33)	<0.001
Serum HBsAg (log_{10} IU/ml)‡	4.62 (4.32, 4.85)	4.11 (3.68, 4.60)	3.23 (2.61, 3.60)	3.43 (3.06, 3.73)	<0.001
Serum anti-HBs (positive/negative, N)‡,§	14/103	32/169	24/144	7/52	0.789
IH HBcAg (positive/negative, N)‡	100/18	160/51	6/168	16/44	<0.001
IH HBsAg (positive/negative, N)‡	115/3	202/9	155/19	57/3	0.005

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ChE, cholinesterase; GGT, gamma-glutamyl transferase; PLT, platelets.
†The phases were determined by the liver histology of these patients, as detailed in the Supplementary material (Patients and methods).
‡Data were presented as number, positive rates or median (interquartile range).
§Lower limit of quantification of serum anti-HBs was 2.00 IU/L. 11 HBeAg-positive patients and seven HBeAg-negative patients were not assessed.
*The two-tailed Mann-Whitney *U* test or X^2 test was performed for comparison between HBeAg-positive and HBeAg-negative groups and p <0.05 was considered statistically significant.

Results

Secretion efficiency of HBsAg is lower in HBeAg-negative patients

To characterise HBsAg expression and secretion patterns in HBeAg-positive and -negative patients, a total of 563 treatment-naive patients with chronic HBV infection were enrolled, including 329 HBeAg-positive and 234 HBeAg-negative patients. Clinical characteristics of the two groups are shown in Table 1. All these patients had serum specimens and corresponding liver biopsy samples available. Immunohistochemistry (IHC) of liver biopsy tissues was conducted, and the protein levels of intrahepatic HBsAg and HBcAg were then semi-quantitatively assayed (Fig. 1A), as described in the Supplementary material (Patients and methods). Consistent with previous studies, HBeAg-positive patients had significantly higher serum HBV DNA levels (8.00 *vs.* 3.36 log_{10} IU/ml, p <0.001) and serum HBsAg levels (4.33 *vs.* 3.30 log_{10} IU/mL, p <0.001) than HBeAg-negative patients (Table 1, Fig. 1B). Unexpectedly, the IHC scores of intrahepatic HBsAg levels were almost identical between the two groups (5.64 *vs.* 5.67 IHC score, p = 0.542), although the proportion of intrahepatic HBsAg-positive being statistically lower in HBeAg-negative patients compared with HBeAg-positive patients (90.60% *vs.* 96.35%, p = 0.005) (Table 1, Fig. 1C). The inconsistency between serum HBsAg and intrahepatic HBsAg levels prompted us to explore if different HBsAg secretion efficiency existed in these HBeAg-positive and -negative patients. A comparison using the ratio of serum HBsAg to intrahepatic HBsAg levels revealed that HBsAg secretion efficiency was significantly lower in HBeAg-negative patients than in HBeAg-positive patients (5.376 *vs.* 6.629, p <0.001, Fig. 1D). We further classified these patients into more precise phases and compared their HBsAg secretion efficiency. The lowest HBsAg secretion efficiency was observed in patients with HBeAg-negative chronic infection, whereas it did not significantly differ from that of HBeAg-negative CHB patients (Fig. 1E). Taken together, these results suggest that lower serum HBsAg levels in HBeAg-negative patients may be partially attributable to decreased secretion efficiency of HBsAg.

HBsAg mainly originates from integrated HBV DNA in HBeAg-negative patients

To determine whether the difference in HBsAg secretion efficiency between HBeAg-positive and -negative patients was as a result of different sources of HBsAg, we first compared intrahepatic HBcAg IHC between the two groups. It revealed that positive rates of intrahepatic HBcAg in HBeAg-negative patients were dramatically lower than that in HBeAg-positive patients (9.40% *vs.* 79.03%, p <0.001) (Table 1). Next, we analysed the correlation between serum HBsAg and two surrogate markers of hepatic cccDNA activity, serum HBV DNA and intrahepatic HBcAg, in HBeAg-positive and -negative patients, respectively. In contrast to the positive correlation between serum HBsAg levels and serum HBV DNA (r = 0.699, p <0.001, Fig. 2A) or intrahepatic HBcAg levels (r = 0.409, p <0.001, Fig. 2B) in HBeAg-positive patients, the correlation became suboptimal for serum HBV DNA levels (r = 0.143, p = 0.029, Fig. 2C) and was absent for intrahepatic HBcAg levels (r = 0.117, p = 0.073, Fig. 2D) in HBeAg-negative patients. This

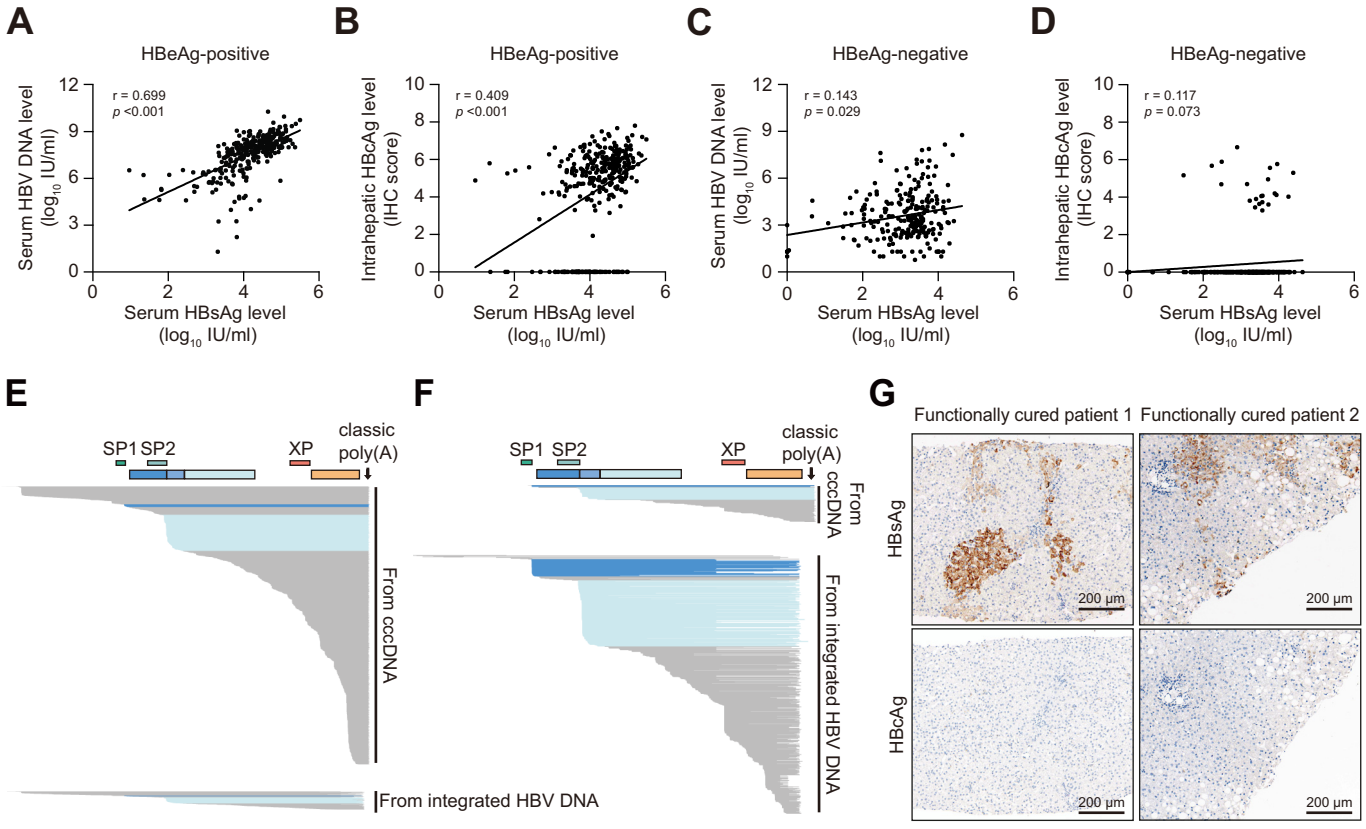

Fig. 2. Integrated HBV DNA was the primary source of HBsAg in HBeAg-negative patients. (A–D) Pearson's correlation analysis was conducted for serum HBsAg levels and serum HBV DNA levels or intrahepatic HBcAg levels in HBeAg-positive patients (A and B) and in HBeAg-negative patients (C and D). (E and F) HBV transcripts detected in liver tissues from one HBeAg-positive patient (E) and one HBeAg-negative patient (F) by long-read RNA sequencing. The lines indicate the part of individual reads that aligned to HBV genome, with blue and cyan representing 2.4 kb RNA and 2.1 kb RNA, respectively. The bar graph showed the corresponding position of HBV promoters and ORFs. (G) Representative IHC images of liver biopsy samples from 'functionally cured' patients stained with anti-HBs and anti-HBc antibodies. (p <0.05 was considered statistically significant). cccDNA, covalently closed circular DNA; ORF, open reading frame; SP1, preS1 promoter; SP2, preS2 promoter; XP, X promoter.

inconsistent correlation strongly indicates that origin of HBsAg is different between the two groups. Indeed, negative intrahepatic HBcAg staining in HBeAg-negative patients implicated the loss or transcription silencing of cccDNA, and integrated HBV DNA might turn to be the major origin of HBsAg in such patients.

To further demonstrate different origins of HBsAg in HBeAg-positive and -negative patients, long-read RNA sequencing analysis was performed on liver tissues of one HBeAg-positive and one HBeAg-negative patient. Based on characteristics of their 3′ tails, the origin of these HBV transcripts was determined. It revealed that 92.44% of viral transcripts originated from cccDNA in the HBeAg-positive patient, whereas 87.65% of viral transcripts originated from integrated HBV DNA in the HBeAg-negative patient (Fig. 2E and F). For HBsAg mRNA, 84.39% of preS/S mRNAs originated from integrated HBV DNA in the HBeAg-negative patient, but this percentage decreased to 12.94% in the HBeAg-positive patient. Consistently, the HBeAg-negative patient had lower intrahepatic cccDNA levels compared to that of the HBeAg-positive patient (109.945 *vs.* 3,719.166 copies/10^5 cells). Taken together, results here indicated that cccDNA was the dominant source of HBsAg in HBeAg-positive patients, while in HBeAg-negative patients, HBsAg is mainly from integrated HBV DNA. Therefore, it is rational to speculate that the lower HBsAg secretion efficiency in HBeAg-negative patients could be attributed to dominant expression of HBsAg from integrated HBV DNA.

HBsAg derived from integrated HBV DNA exhibits low secretion efficiency

In line with the low secretion efficiency of HBsAg observed in HBeAg-negative patients, in some functionally cured patients with CHB (12/62) undergoing Peg-IFNα-based treatment, their intrahepatic HBsAg staining were still positive despite being negative for serum HBsAg (Table S1, Fig. 2G, and Fig. S1). Moreover, residual intrahepatic HBsAg in these patients mainly originated from integrated HBV DNA.[13] Thus, potential HBsAg-secretion deficiency in these 'functionally cured' patients suggested that HBsAg derived from integrated HBV DNA might exhibit low secretion efficiency.

To characterise universal sequence features of integrated HBV DNA, a large number of HBV integration breakpoints were collected from the Viral Integration Site Database (Table S2). Most integrated HBV DNA fragments were found to be terminated at two ends of dslDNA, particularly in a narrowed region surrounding the DR1 (Fig. 3A). Therefore, we constructed a dslDNA expression plasmid (p-dslDNA) to simulate common sequence features of integrated HBV DNA fragments in patients with CHB, as detailed in the Supplementary methods.

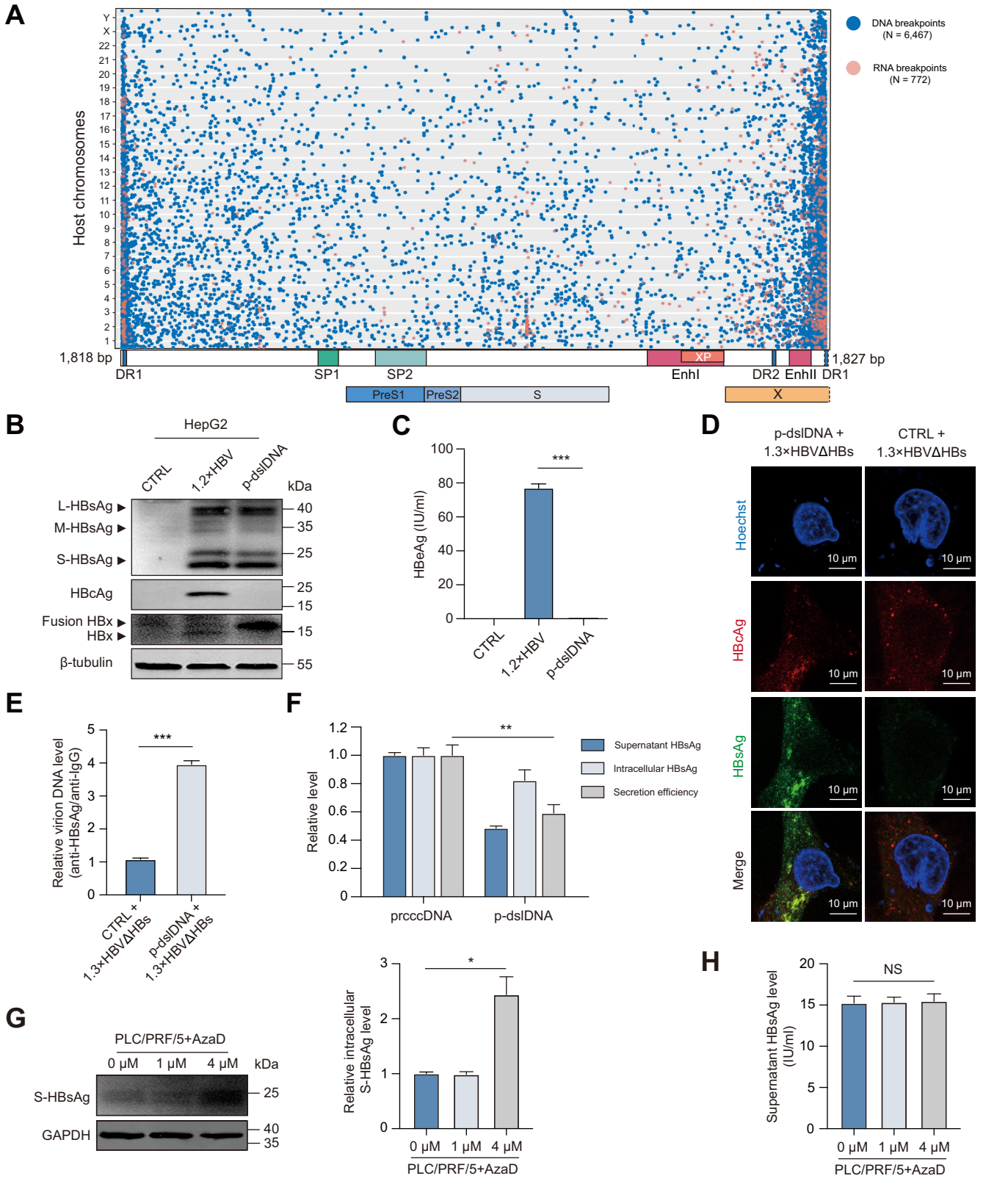

Fig. 3. The secretion of HBsAg derived from integrated HBV DNA was inhibited. (A) HBV breakpoints collected from Virus Integration Site DataBase (VISDB) and mapped across HBV dslDNA (1818∼3215/0∼1827 bp) sequence (x-axis) and human genome (y-axis) respectively. Blue dots represented HBV DNA breakpoints, red dots represented HBV RNA breakpoints. (B and C) HepG2 cells were transfected with p-dslDNA, a vector control, or a positive control (1.2 × HBV plasmid). At 72 h post-transfection, L-/M-/S-HBsAg, HBcAg, and HBx levels were detected by Western blotting (B), HBeAg levels were determined by CLIA (C). (D and E) HepG2 cells were co-transfected with 1.3 × HBVΔHBs and p-dslDNA (or vector control). At 72 h post-transfection, the localisation of HBsAg (green) and HBcAg (red) was evaluated

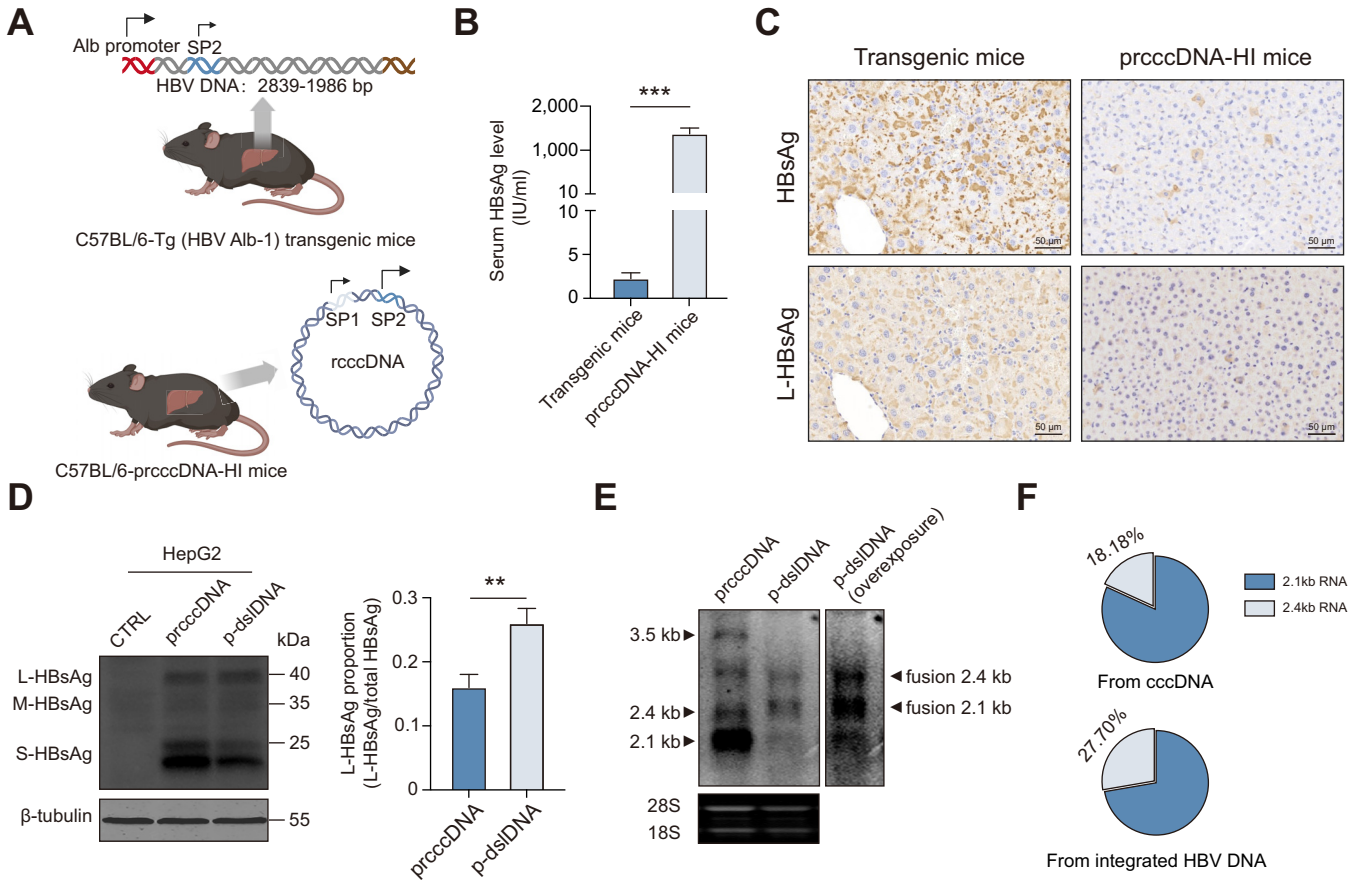

Fig. 4. Integrated HBV DNA expressed a higher proportion of L-HBsAg than cccDNA. (A) Schematic diagram of C57BL/6-Tg (HBV Alb-1) transgenic mice and C57BL/6-prcccDNA-HI mice. (B) Serum HBsAg levels were compared between transgenic mice (N = 4) and prcccDNA-HI mice (N = 4). (C) Representative IHC images of liver tissues from transgenic mice and prcccDNA-HI mice stained with anti-HBs and anti-preS1 antibodies. (D and E) HepG2 cells were transfected with equal copies of prcccDNA (along with pCMV-Cre) or p-dslDNA. At 72 h post-transfection, intracellular HBsAg levels were determined by Western blotting (D) and intracellular HBV RNA levels were detected by Northern blotting (E). Grey value analysis was performed on L-HBsAg and total HBsAg levels relative to β-tubulin levels, the proportion of intracellular L-HBsAg to total HBsAg was then calculated (D, right). (F) The proportion of preS/S mRNAs transcribed from cccDNA (upper) or from integrated HBV DNA (lower) in the HBeAg-negative patient (Fig. 2F). (The data are shown as the mean ± SD, two-tailed Student *t* test, ***p* <0.01, ****p* <0.001). cccDNA, covalently closed circular DNA.

When transfected into HepG2 cells, p-dslDNA expressed L-, M-, S-HBsAg and fusion HBx (Fig. 3B and Fig. S2A). As expected, HBcAg and HBeAg were not detected because of partial truncation of the CP promoter sequence (Fig. 3B and C). Additionally, HBsAg derived from p-dslDNA could support assembly of Dane particles derived from 1.3 × HBVΔHBs (Fig. S2B–D), which was confirmed by the co-localisation of HBcAg and HBsAg in cytoplasm (Fig. 3D), and the upregulation of virion-associated HBV DNA levels in supernatant as demonstrated by quantitative PCR (qPCR) measurement of enveloped HBV DNA purified by anti-S/preS2 antibody pull-down (Fig. 3E). Together, p-dslDNA can simulate the expression of common integrated HBV DNA fragments.

To confirm the different secretion efficiency of HBsAg between integrated HBV DNA and cccDNA, equal copies of precursor plasmid bearing a recombinant cccDNA (prcccDNA)[14] and p-dslDNA were transiently transfected into HepG2 cells to simulate cccDNA and integrated HBV DNA, respectively. The HBsAg secretion efficiency, measured by the ratio of quantitatively measured HBsAg in supernatant to that in cell lysates, was ∼40% lower in p-dslDNA group compared with the prcccDNA group (Fig. 3F). Next, PLC/PRF/5 a human hepatoma cell line containing natural HBV integrations was used to further confirm the observation of impaired HBsAg secretion efficiency derived from integrated HBV DNA. As the expression of integrated HBV DNA was partially silenced by DNA methylation in PLC/PRF/5,[15] cells were firstly treated with 5-aza-2′-deoxycytidine (AzaD) for demethylation. The respective changes in intracellular and secreted HBsAg were then quantitatively analysed. As shown in Fig. 3G, an approximate

by immunostaining (D), HBV DNA levels within HBV virions were detected by immunoprecipitation-qPCR (E). (F) HepG2 cells were transfected with equal copies of prcccDNA (along with pCMV-Cre) or p-dslDNA. At 72 h post-transfection, the levels of secreted HBsAg and intracellular HBsAg were detected by CLIA, the HBsAg secretion efficiency were then calculated. (G and H) PLC/PRF/5 cells were treated with different AzaD concentrations, 72 h post-treatment, the levels of intracellular and secreted HBsAg were detected by Western blotting (G) and CLIA (H), respectively. Grey value analysis was performed on cellular S-HBsAg levels relative to GAPDH levels. (Two-tailed Student *t* test, * *p* <0.05, ** *p* <0.01, *** *p* <0.001, NS, not significant.) AzaD, 5-aza-2′-deoxycytidine; CLIA, chemiluminescence immunoassay; EnhI, HBV enhancer 1; EnhII, HBV enhancer 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SP1, preS1 promoter; SP2, preS2 promoter.

two-fold increase of intracellular HBsAg levels was observed after treatment with 4 μM AzaD. In contrast, no observable changes of HBsAg in supernatant occurred (Fig. 3H). The inconsistent dynamics of intracellular and secreted HBsAg levels further indicated that the impaired secretion of HBsAg originated from integrated HBV DNA.

A high proportion of L-HBsAg inhibits the secretion of HBsAg derived from integrated HBV DNA

As a high proportion of L-HBsAg would impact the secretion of total HBsAg,[16] we wondered if the low HBsAg secretion efficiency of integrated HBV DNA was caused by excessive expression of L-HBsAg. Because the integrated HBV DNA fragment contains intact preS/S ORF but with the SP1 promoter replaced by a much stronger promoter of the Alb-1 gene in C57BL/6-Tg (HBV Alb-1) transgenic mice (Fig. 4A), the model was used for further study. Compared with prcccDNA-HI mice, significantly lower serum HBsAg levels, as well as much higher intrahepatic HBsAg levels were observed in transgenic mice (Fig. 4B and C), implicating the deficiency of HBsAg secretion in transgenic mice. Meanwhile, we noticed strong *in situ* L-HBsAg expression in liver tissues of transgenic mice (Fig. 4C). These results indicated that the HBsAg secretion of integrated HBV DNA would be impaired when L-HBsAg proportion increased.

Next, Western blotting analysis was conducted to investigate the HBsAg composition of p-dslDNA-transfected HepG2 cells with lower HBsAg secretion efficiency. As expected, there was a significant increase in the proportion of intracellular L-HBsAg observed in p-dslDNA-transfected cells (Fig. 4D). Mechanistically, as revealed by Northern blotting analysis, a

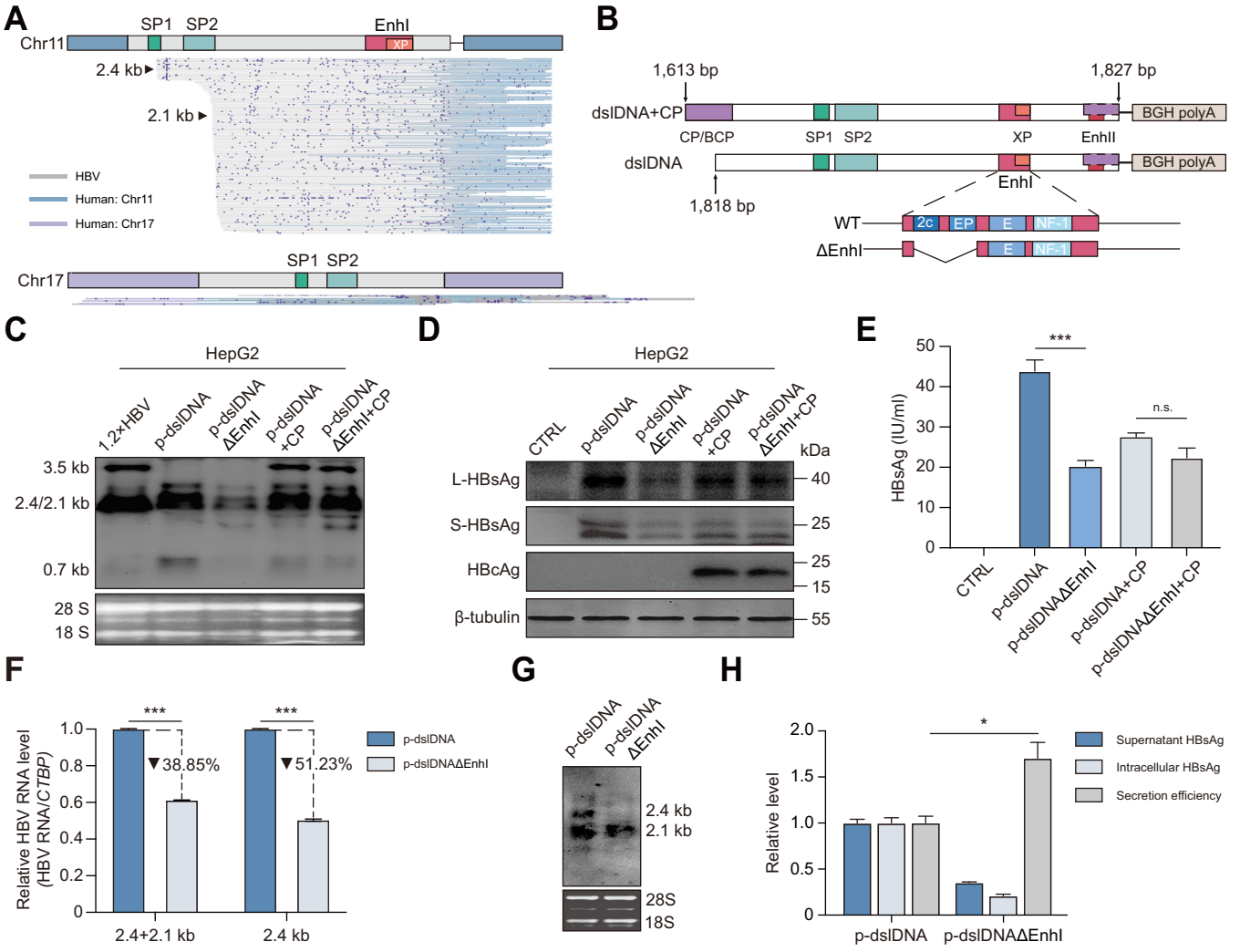

Fig. 5. EnhI re-targeted SP1 and SP2 promoters when CP promoter was deficient. (A) Fusion HBV-host transcripts derived from the integrated HBV DNA fragment on chromosome 11 or 17 of PLC/PRF/5 cells. The lines indicate individual fusion transcripts. The upper bar graph shows the corresponding position of HBV promoters and enhancer. (B) Pattern diagram of dslDNA+CP and dslDNAΔEnhI plasmids. (C-E) HepG2 cells were transfected with 1.2 × HBV (positive control), p-dslDNA, p-dslDNAΔEnhI, p-dslDNA+CP or p-dslDNAΔEnhI+CP. At 72 h post-transfection, intracellular HBV RNA levels were determined by Northern blotting (C), L-/S-HBsAg and HBcAg levels were detected by Western blotting (D), secreted HBsAg levels were assessed by CLIA (E). (F–H) HepG2 cells were transfected with p-dslDNA or p-dslDNAΔEnhI, 72 h post-transfection, intracellular preS/S mRNAs levels were determined by RT-qPCR (F) and northern blotting (G). The levels of secreted and intracellular HBsAg were detected by CLIA, then HBsAg secretion efficiency was calculated (H). (Two-tailed Student *t* test, **p* <0.05, ****p* <0.001, NS, not significant.) CLIA, chemiluminescence immunoassay; CP, core promoter; EnhI, HBV enhancer 1; SP1, preS1 promoter; SP2, preS2 promoter; WT, wild-type.

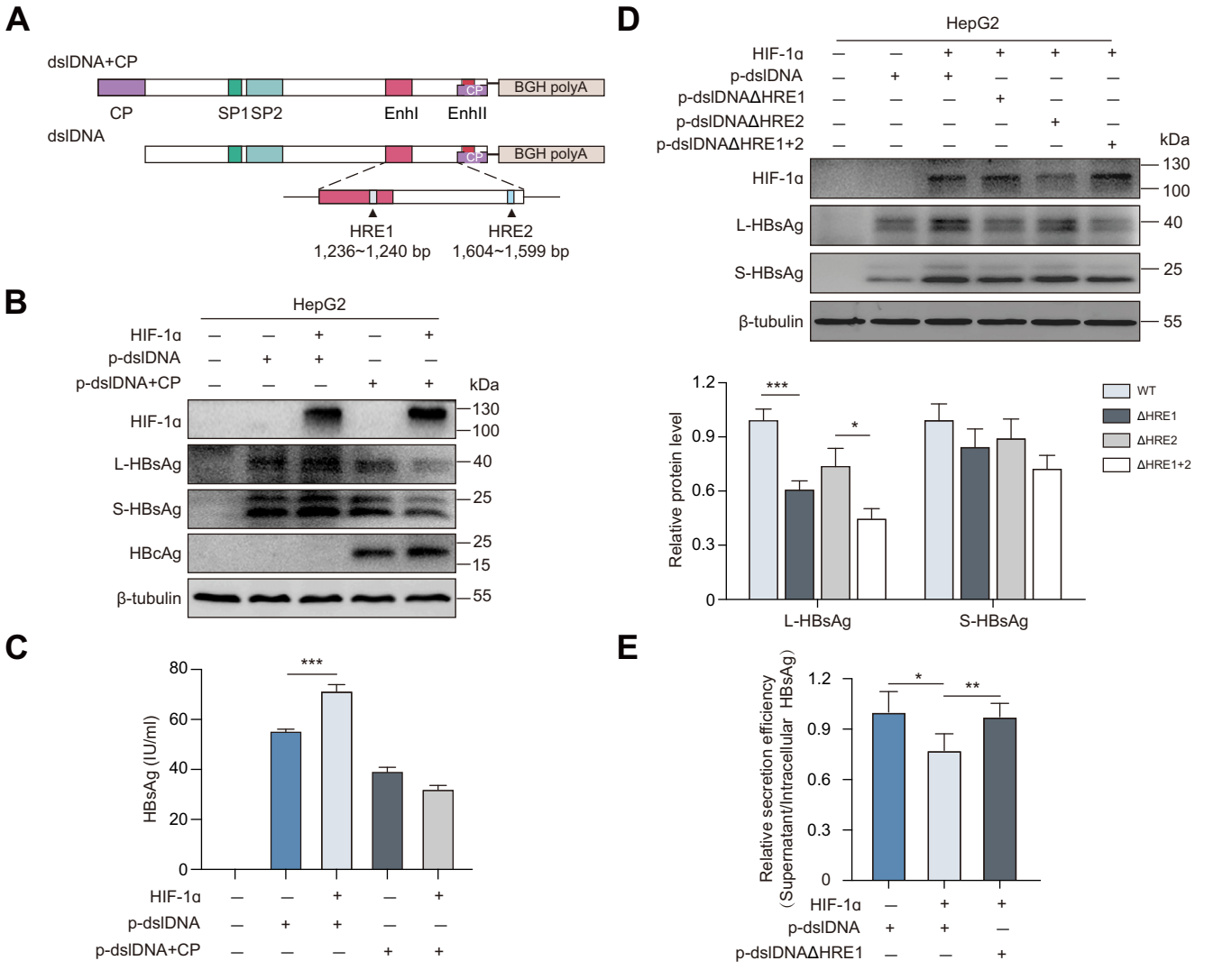

Fig. 6. HIF-1α regulated HBsAg expression through the HRE1 located within EnhI when CP promoter was deficient. (A) Pattern diagram of HREs distribution in HBV genome. (B and C) HepG2 cells were co-transfected with an HIF-1α expression plasmid (or vector control) and p-dslDNA or p-dslDNA+CP. At 72 h post-transfection, HIF-1α, L-/S-HBsAg, and HBcAg levels were detected by Western blotting (B), secreted HBsAg levels were assessed by CLIA (C). (D) HepG2 cells were co-transfected with an HIF-1α expression plasmid and p-dslDNA, p-dslDNAΔHRE1, p-dslDNAΔHRE2, or p-dslDNAΔHRE1+2. At 72 h post-transfection, HIF-1α and L-/S-HBsAg levels were detected by Western blotting. Grey value analysis was performed on the levels of L-HBsAg and S-HBsAg relative to β-tubulin levels. (E) HepG2 cells were co-transfected with an HIF-1α expression plasmid (or vector control) and p-dslDNA, p-dslDNAΔHRE1. At 72 h post-transfection, secreted and intracellular HBsAg levels were detected by CLIA, HBsAg secretion efficiency was then calculated. (Two-tailed Student *t* test, **p* <0.05, ***p* <0.01, ****p* <0.001.) CP, core promoter; EnhI, HBV enhancer 1; EnhII, HBV enhancer 2; HIF-1α, hypoxia inducible factor-1α; HRE, hypoxia response element; SP1, preS1 promoter; SP2, preS2 promoter; WT, wild-type.

higher proportion of 2.4 kb RNA to total preS/S mRNAs in the p-dslDNA-transfected group was observed, as compared with the prcccDNA-transfected group (Fig. 4E). A noticeably longer HBV RNA derived from p-dslDNA was a result of the presence of an additional skeleton sequence between the HBV sequence and the BGH poly(A) signal. Additionally, the higher HBsAg secretion efficiency in prcccDNA group could be a result of its ability to secrete more HBsAg along with the release of Dane particles. To exclude the possibility, we introduced a HBcAg premature stop codon into the wild-type prcccDNA to prevent the assembly of Dane particles (Fig. S3A), and no impact of HBsAg secretion efficiency was observed between wild-type prcccDNA and prcccDNAΔHBc/HBe groups (Fig. S3B–D). Importantly, the proportion of 2.4 kb RNA derived from integrated HBV DNA was higher than that derived from cccDNA (27.70% *vs.* 18.18%), demonstrated by long-read RNA sequencing of a liver biopsy sample from the HBeAg-negative patient (Figs. 2F and 4F). In summary, the high proportion of 2.4 kb RNA derived from integrated HBV DNA can lead to excessive L-HBsAg, which might subsequently result in low HBsAg secretion efficiency in HBeAg-negative patients.

EnhI re-targets SP1 and SP2 promoters then unevenly activates SP1 in integrated HBV DNA

It is well known that the sequence of CP promoter is partially truncated in integrated HBV DNA, whereas its partner enhancer EnhI is fully preserved in most integrated HBV DNA fragments

(Fig. 3A). A mechanism known as 'enhancer release and retargeting' has been reported recently in mammalian cells.[11] We questioned if, because of the loss of the partner promoter, EnhI would retarget and transcriptionally regulate the activity of SP1 and SP2 promoters in the same mechanism.

First, based on the data of long-read DNA sequencing on PLC/PRF/5 cells in our previous study,[17] we found that integrated HBV DNA fragment on chromosome 11 retained both SP1, SP2, and EnhI, whereas the integrated HBV DNA fragment on chromosome 17 only retained SP1 and SP2 but not EnhI, which made it an ideal cell model to explore the role of EnhI in integrated HBV DNA. Then, long-read RNA sequencing on PLC/PRF/5 cells was performed. The transcriptional activity of the integrated HBV DNA fragment on chromosome 11 was significantly higher than that on chromosome 17 (Fig. 5A). In contrast to chromosome 11, there was no 2.4 kb RNA transcript derived from the integrated HBV DNA fragment on chromosome 17 (Fig. 5A). These results suggest that EnhI plays an important role in transcriptional regulation of integrated HBV DNA.

To further investigate whether EnhI is involved in the regulation of HBsAg expression in integrated HBV DNA, we mutated EnhI by deleting the 50 bp HBV sequence (1,117∼1,166 bp) within core region of EnhI,[18] but outside the X promoter region, from p-dslDNA (p-dslDNAΔEnhI) and 1.2 × HBV plasmid (1.2 × HBVΔEnhI) (Fig. 5B). In contrast to slight changes of intracellular and supernatant HBsAg levels in 1.2 × HBVΔEnhI (Fig. S4A and B), HBsAg levels were significantly reduced when the same region of EnhI was deleted in p-dslDNA (Fig. S4C and D). These results suggest that EnhI might be specifically associated with the regulation of HBsAg expression in integrated HBV DNA. To further confirm whether HBsAg production in integrated HBV DNA is associated with the 'enhancer release and retargeting' mechanism, an intact CP promoter was added at the 5′ end of p-dslDNA and p-dslDNAΔEnhI (Fig. 5B). Transient transfection assays showed that restoration of the CP promoter resulted in the recovery of 3.5 kb RNA transcription and HBcAg expression (Fig. 5C–E). Notably, in contrast to the significant reduction of preS/S mRNAs level in p-dslDNA (Fig. 5C, lane 2 *vs.* 3), the deletion mutation of EnhI did not influence preS/S mRNAs level in the presence of a functional CP promoter (Fig. 5C, lane 4 *vs.* 5). Consistently, EnhI mutation significantly decreased both intracellular and secreted HBsAg protein levels in the case of a deficient CP promoter (Fig. 5D and E, lane 2 *vs.* 3), whereas such decrease was not observed in the presence of a functional CP promoter (Fig. 5D and E, lane 4 *vs.* 5). These results indicate that EnhI selectively activates SP1 and SP2 promoters in integrated HBV DNA with CP promoter truncation.

To explore whether EnhI disrupted the balance of transcriptional activity between SP1 and SP2 promoters, the core region of EnhI was deleted from p-dslDNA, then the levels of 2.4 kb RNA and total preS/S mRNAs were measured and compared. The results demonstrated that EnhI had a stronger activation on SP1 than SP2 in p-dslDNA, as the mutation of EnhI primarily led to a reduction in 2.4 kb RNA level (Fig. 5F and G). In line with this, HBsAg secretion efficiency increased by nearly two-fold when EnhI mutation occurred (Fig. 5H). Collectively, the functional loss of the CP promoter in integrated HBV DNA may lead to EnhI retarget SP1 and SP2 promoters and disrupt their transcriptional activity balance, resulting in over-expression of L-HBsAg which in turn inhibited HBsAg secretion.

Activated EnhI by HIF-1α can unevenly enhance SP1 and SP2 promoters in the case of a deficient CP promoter

To confirm the selective regulation of EnhI to SP1 and SP2 promoters in the case of a deficient CP promoter, we overexpressed hypoxia inducible factor-1α (HIF-1α), which was previously reported to bind to hypoxia response element (HRE) in the HBV genome (Fig. 6A) and promote HBV replication.[19] Results showed that HIF-1α upregulated both intracellular and secreted HBsAg levels in the p-dslDNA group (Fig. 6B and C, lane 2 *vs.* 3). Meanwhile, in the p-dslDNA+CP group, HIF-1α mainly upregulated the levels of HBcAg, which was translated from the 3.5 kb RNA promoted by CP promoter, instead of HBsAg levels (Fig. 6B and C, lane 4 *vs.* 5). Similarly, HIF-1α could upregulate HBcAg of 1.2 × HBV with intact CP promoter, instead of HBsAg levels (Fig. S5A and B). Considering that there are two HREs in the HBV genome (Fig. 6A) and to confirm that the HRE1 located in EnhI sequence was responsible for alternative upregulation of HBsAg from p-dslDNA by HIF-1α, we next constructed an HRE1-deletion mutation (p-dslDNAΔHRE1), as well as p-dslDNAΔHRE2 and mutation with jointly deleted HREs (p-dslDNAΔHRE1+2). After confirming that the 5∼10 bp deletion did not affect HBsAg expression in the absence of HIF-1α (Fig. S5C), the wild-type or HRE-deleted p-dslDNA was co-transfected with HIF-1α expression plasmid into HepG2 cells. Compared with HRE2 deletion, HRE1 deletion substantially inhibited the upregulation of HBsAg levels by HIF-1α (Fig. 6D). These results suggested that HIF-1α could selectively upregulate the HBsAg level through HRE1 located in EnhI in integrated HBV DNA. Notably, HRE1 deletion significantly inhibited the upregulation of L-HBsAg expression by HIF-1α, compared with S-HBsAg expression (Fig. 6D), which further supported the discovery that EnhI had a stronger activation on SP1 than SP2 promoter. Consistently, when co-transfecting p-dslDNA or p-dslDNA with HRE1 deletion (p-dslDNAΔHRE1) and HIF-1α expression plasmids, the HBsAg secretion efficiency of p-dslDNA slightly decreased but was partially rescued when transfecting p-dslDNAΔHRE1 (Fig. 6E). Collectively, when activated by HIF-1α, EnhI could further disrupt the transcriptional activity balance between SP1 and SP2 promoters in the case of a deficient CP promoter. The overexpression of L-HBsAg subsequently reduced HBsAg secretion efficiency.

Discussion

Although it has become gradually accepted that integrated HBV DNA serves as an important source of serum HBsAg,[5] the expression and secretion characteristics of HBsAg derived from integrated HBV DNA, as well as the potential regulation mechanisms remain unclear. Here, we explored the regulation mechanism of HBsAg expression that originated from integrated HBV DNA and characterised its secretion pattern in clinical cohorts, an *in vitro* model, and HBV transgenic mice. Of note, the secretion efficiency of HBsAg derived from integrated HBV DNA was lower than that from cccDNA. Mechanistically, functional deficiency of CP promoter in integrated HBV DNA leads to selective activation of SP1 and SP2 promoters by EnhI, which results in a higher proportion of L-HBsAg expression.

It has previously been reported that integrated HBV DNA is the dominant origin of HBsAg in HBeAg-negative chimpanzees.[20] In addition, results of targeted Iso-Seq of liver biopsy samples from patients with CHB revealed that HBV transcripts

were mainly transcribed from integrated HBV DNA in HBeAg-negative patients.[8] Similarly, in our third-generation sequencing results, up to 84.39% of preS/S mRNAs were transcribed from integrated HBV DNA in one HBeAg-negative patient, compared with only 12.94% in one HBeAg-positive patient. These observations strongly reinforce the hypothesis that integrated HBV DNA is the dominant source of HBsAg in HBeAg-negative patients.

Interestingly, we found that although serum HBsAg levels were significantly lower in HBeAg-negative patients than that in HBeAg-positive patients, they had similar intrahepatic HBsAg levels. In 1973, hepatocytes with much cytoplasmic HBsAg were observed in inactive HBV carriers.[21] Moreover, the comparable intrahepatic HBsAg-positive rate between HBeAg-positive and -negative patients were recently reported based on a novel four-plex immunofluorescence assay and image analysis.[22] Such phenomena prompted us to demonstrate whether the HBsAg secretion efficiency of integrated HBV DNA differed from cccDNA which has not been reported in literature before. In this study, we revealed that HBsAg secretion efficiency was significantly lower in HBeAg-negative patients than that in HBeAg-positive patients based on the ratio of serum HBsAg and intrahepatic HBsAg, which might limit the accuracy. Furthermore, our *in vitro* studies in cell culture and transgenic mice confirmed the clinical phenomenon. The quantification of secreted HBsAg and intracellular HBsAg was measured by the same approach in those cell culture experiments. Consistently, HBsAg secretion efficiency was significantly lower in integrated HBV DNA mimicking p-dslDNA group than in cccDNA-mimicking prcccDNA group. Moreover, RNA-seq assays of liver biopsy tissues were previously performed on 'functionally cured' patients with positive intracellular HBsAg staining in our study cohort.[13] The results indicated that residual intrahepatic HBsAg in such 'functionally cured' patients were mainly from integrated HBV DNA, which provided an additional support to our finding that HBsAg derived from integrated HBV DNA exhibited low secretion efficiency.

Several studies have reported that a high proportion of L-HBsAg could inhibit HBsAg secretion.[23,24] Elevated L-HBsAg expression and decreased S-HBsAg expression was previously observed in cells transfected with integrated HBV DNA from PLC/PRF/5 cells.[25] Yen *et al.*[26] also revealed that in contrast to S-HBsAg, high levels of L-HBsAg are sustained in HBV-related HCC samples. Integrated HBV DNA can generate ~80% of HBsAg transcripts in HBV-related HCC samples.[27] Therefore, it is rational to hypothesise that lower HBsAg secretion efficiency seen in HBeAg-negative patients may be attributed to the excessive expression of L-HBsAg from integrated HBV DNA. To further verify the above hypothesis, we constructed an *in vitro* model to mimic common sequence features of naturally existing integrated HBV DNA fragments.[28] Compared with the cccDNA-simulating construct,[14] cells transfected with p-dslDNA had lower HBsAg secretion efficiency. Meanwhile, p-dslDNA had higher proportion of 2.4 kb RNA as well as consequently higher ratio of L-HBsAg to total HBsAg. These *in vitro* results were confirmed by long-read RNA sequencing analysis, and there was a higher proportion of 2.4 kb RNA from integrated HBV DNA than cccDNA in the HBeAg-negative patient; however, a larger cohort would be required for further verification. Although a high proportion of L-HBsAg could inhibit the secretion of virions and subviral particles (SVPs),[29] the low HBsAg secretion efficiency observed in HBeAg-negative patients might be mainly attributed to decreased release of SVPs, because SVPs outnumber Dane particles by 10^3-fold or more *in vivo*.[30]

In addition, a previous study revealed that histone post-translational modifications of cccDNA were significantly different between HBeAg-positive and -negative patients.[31] Our recent study further revealed that in contrast to HBeAg-positive patients, SP2 promoter region of cccDNA in HBeAg-negative patients had low levels of H3K4me3, which is commonly associated with the activation of transcription of nearby genes. Meanwhile, H3K4me3 was highly enriched around SP1 over SP2 promoters of integrated HBV DNA in PLC/PRF/5 cells.[32] These results suggested that SP2 promoter of both cccDNA and integrated HBV DNA were transcriptionally inhibited in HBeAg-negative patients, which might further lead to a relatively high proportion of 2.4 kb RNA to inhibit HBsAg secretion.

In integrated HBV DNA, CP promoter is non-functional, whereas its partner enhancer, EnhI, was reported to be conserved and functionally active in integrated HBV DNA of Hep3B and PLC/PRF/5 cells.[33] This prompted us to hypothesise that an 'enhancer release and retargeting' mechanism[11] discovered in mammalian cells might play a role in excessive L-HBsAg expression seen in integrated HBV DNA. Consistently, long-read RNA sequencing analysis of PLC/PRF/5 cells confirmed selective enhancement of SP1 and SP2 promoters by EnhI in integrated HBV DNA. Based on a p-dslDNA model, we then demonstrated that the EnhI element could selectively activate SP1 and SP2 promoters when the CP promoter is truncated, through an 'enhancer release and retargeting' mechanism. In line with this, HIF-1α, which was previously reported to activate the CP promoter through HREs in the HBV genome,[19] was able to selectively upregulate HBsAg levels through HRE1 located within EnhI in the absence of a functional CP promoter. Moreover, HRE1 deletion in the EnhI sequence resulted in a more significant inhibitory effect of HIF-1α on L-HBsAg than on S-HBsAg levels. Consistently, compared with 2.1 kb RNA, deletion mutation of EnhI also predominantly reduced 2.4 kb RNA transcription. All these results suggested that EnhI preferentially activates SP1 rather than SP2 promoter, which may partially explain why integrated HBV DNA expresses a high L-HBsAg proportion. It is well known that CCCTC-binding factor (CTCF) orchestrates the interactions between enhancers and promoters.[34] In the HBV genome, there are two identified CTCF-binding sites, including one located in EnhI region and another located between SP1 and CP promoter.[35] Here, we reasoned that the interactions between EnhI and SP1 promoter in integrated HBV DNA may be mediated by CTCF, which requires further validation.

In summary, we revealed that functional deficiency of CP promoter leads to uneven activation of SP1 and SP2 promoters by EnhI, thus disrupts the transcriptional activity balance between SP1 and SP2 promoters in integrated HBV DNA. In HBeAg-negative patients, the high proportion of L-HBsAg derived from integrated HBV DNA might inhibit HBsAg secretion. These findings expand our understanding of HBV integration which might pave the way for a functional cure of CHB.

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Abbreviations

AzaD, 5-aza-2′-deoxycytidine; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CP, core promoter; CTCF, CCCTC-binding factor; dslDNA, double strand linear DNA; EnhI, HBV enhancer 1, EnhII, HBV enhancer 2; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, hepatitis B virus protein x, HCC, hepatocellular carcinoma; HIF-1α, hypoxia inducible factor-1α; HRE, hypoxia response element; IHC, immunohistochemistry; L-HBsAg, large hepatitis B surface antigen; NAs, nucleos(t)ide analogues; ORF, open reading frame; p-dslDNA, dslDNA expression plasmid; Peg-IFNα, pegylated interferon α; prcccDNA, precursor plasmid bearing a recombinant cccDNA; qPCR, quantitative PCR; SP1, preS1 promoter; SP2, preS2 promoter; SVPs, subviral particles.

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Conflicts of interest

All authors disclose no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design and interpretation of data: FL, XC, HH, JZ, ZGa, ZZ, SL. Drafting the manuscript: FL, XC, ZGu, HH, QJ, AA. Experiments: ZGu, QJ, XC, ML, NG, TZ, DY, JX, GY. Acquisition of data: ML, ZGu, XC, NG. Data analysis: AA, ZGu, XC, GG. Obtained funding: FL, XC.

Data availability statement

The long-read RNA sequencing data of PLC/PRF/5 cells have been uploaded to the NCBI database (PRJNA1108239), and raw data of liver biopsy samples from patients with CHB are available and can be reviewed upon request.

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Supplementary data

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