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High mobility group AT-hook 1 (HMGA1) is an important positive regulator of hepatitis B virus (HBV) that is reciprocally upregulated by HBV X protein

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ABSTRACT

Chronic infection with hepatitis B virus (HBV) is associated with liver cirrhosis and hepatocellular carcinoma. Upon infection of hepatocytes, HBV covalently closed circular DNA (cccDNA) exists as histone-bound mini-chromosome, subjected to transcriptional regulation similar to chromosomal DNA. Here we identify high mobility group AT-hook 1 (HMGA1) protein as a positive regulator of HBV transcription that binds to a conserved ATTGG site within enhancer II/core promoter (EII/Cp) and recruits transcription factors FOXO3α and PGC1α. HMGA1-mediated upregulation of EII/Cp results in enhanced viral gene expression and genome replication. Notably, expression of endogenous HMGA1 was also demonstrated to be upregulated by HBV, which involves HBV X protein (HBx) interacting with SP1 transcription factor to activate HMGA1 promoter. Consistent with these *in vitro* results, chronic hepatitis B patients in immune tolerant phase display both higher intrahepatic HMGA1 protein levels and higher serum HBV markers compared to patients in inactive carrier phase. Finally, using a mouse model of HBV persistence, we show that targeting endogenous HMGA1 through RNA interference facilitated HBV clearance. These data establish HMGA1 as an important positive regulator of HBV that is reciprocally upregulated by HBV via HBx and also suggest the HMGA1-HBV positive feedback loop as a potential therapeutic target.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection (CHB) currently affects >250 million people worldwide. In addition to chronic active hepatitis, CHB is associated with high risks of liver cirrhosis and hepatocellular carcinoma, causing ∼0.9 million related deaths annually (http://www.who.int/en/news-room/fact-sheets/detail/hepatitis-b). CHB natural history is traditionally characterized into four phases based on different viral and host immune activity status: immune tolerant (IT) phase, with high viral replication as reflected in high serum HBV DNA, but low or no inflammation in liver as reflected in generally normal serum alanine aminotransferase (ALT) levels; HBV e antigen (HBeAg) positive immune active phase (IA) with both high viral replication and prominent host inflammatory response (hepatitis); inactive CHB or inactive carrier phase (IC) wherein viral replication is low or even undetectable, accompanied by low or marginal ongoing liver inflammation; and HBeAg-negative

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immune reactivation phase, characterized by both reactivated viral replication and exacerbated hepatitis (1). In both IT and IC phases, the immune system is not actively attacking HBV-infected hepatocytes, yet serum HBV DNA levels are usually markedly higher in IT phase compared to IC phase (2). Mechanisms underlying such a difference are not fully clear.

HBV is the type member of *Hepadnaviridae* and is exclusively hepatotropic (3). Upon HBV's entry into hepatocytes through receptor NTCP (4), its ∼3.2 kb partially double-stranded relaxed circular DNA (rcDNA) genome is converted into covalently closed circular DNA (cccDNA) in the nucleus (3,5). Viral transcription uses cccDNA as the only template and is driven by four viral promoter/enhancer elements, namely enhancer II/core antigen (HBcAg) promoter (EII/Cp), surface antigen promoters 1 and 2 (Sp1 and Sp2) and enhancer I/X protein (HBx) promoter (EI/Xp), to produce 3.5, 2.4, 2.1 and 0.7 kb RNA products, respectively (6). The 3.5 kb RNAs function as templates for translation of HBeAg, HBcAg and polymerase (P), and also can serve as pregenomic RNA (pgRNA) that is bound by the translated polymerase *in cis* to initiate reverse transcription. The polymerase-pgRNA complex is encapsidated by core proteins and further reverse transcription and synthesis of positive sense DNA take place inside the capsids. Mature capsids could either be enveloped by large, middle and small surface antigens (L/M/SHBsAg) translated from the 2.4 and 2.1 kb viral mRNAs or be recycled to the nucleus to supplement nuclear cccDNA pool (3,5). The 0.7 kb RNA is translated to produce HBx, which has been shown to be capable of affecting various key viral and cellular processes through interactions with a multitude of cellular factors (7,8).

In the nucleus, HBV cccDNA exists as histone-bound mini-chromosomes (9,10). Transcription is catalyzed by canonical cellular transcription machinery and subjected to epigenetic regulation of transcriptional activity similar to host cell chromosomes (11–13). As initiators of both gene expression and genome replication, HBV enhancer/promoter elements, especially the pgRNA-producing EII/Cp, have been shown to be under complex regulation involving multiple host factors as well as HBx (6,14).

High mobility group (HMG) proteins are small, abundant, chromatin-associated DNA-binding proteins that bend target DNA to modify chromatin structure (15,16). The HMG AT hook (HMGA) family proteins bind B-form DNA in the minor groove at AT-rich sites with sequence-specificity (17). HMGA-binding induces DNA bending, displaces histone H1 and 'widens' the minor groove to facilitate further recruitment of transcription factors and chromatin structure remodelers, usually resulting in transcriptional activation (15,17). *HMGA1* gene is highly expressed during development and plays important roles in regulating proliferation and differentiation (15,18).

In this work, characterization of a highly conserved positive regulatory element within EII/Cp led to the identification of HMGA1 as the key binding factor. We explored the mechanisms of HMGA1-mediated activation of EII/Cp and demonstrated the enhancing effects of such activation on viral gene expression and genome replication. Notably, it was also discovered that HBV upregulates expression of endogenous HMGA1 and the underlying mechanisms were also probed. Since these data indicated the existence of a positive feedback loop between HBV and HMGA1, possible correlation between HBV activities and endogenous HMGA1 levels were examined by comparing CHB patients in IT phase versus patients in IC phase. Finally, importance of HMGA1 for HBV life cycle was tested *in vivo* using a mouse model of HBV persistence.

MATERIALS AND METHODS

Cells

Human hepatocellular carcinoma (Huh7 and HepG2), embryonic kidney (HEK293T) and cervical carcinoma (HeLa), mouse hepatocellular carcinoma (Hepa1-6) cell lines and HepG2 stably transfected with HBV genome (HepAD38) were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 2 mM L-glutamine, 50 U/ml penicillin and 10% fetal bovine serum (all from Invitrogen, China). HepG2 cells stably transfected with human NTCP (HepG2/NTCP) (19) were additionally supplemented with 2.5% dimethyl sulfoxide (DMSO) (Sigma, China) and 2 μg/ml puromycin (Invitrogen, China). Primary human hepatocytes (PHH) cells were purchased from Shanghai RILD Inc. (Shanghai, China) and cultured as previously described (20).

Plasmids, transfections and reporter assay

HBV EII/Cp reporter plasmids (pCp) of genotypes A, B, D, E, H (GenBank Accession Numbers AP007263.1, KR232337, V01460, X75664.1, AB516393.1) and C (21) were constructed by inserting corresponding EII/Cp sequences (nt -171 to + 36 relative to preC) into pGL3-basic (Promega, China). Genotype B Sp1 (nt -629 to -68 relative to preS1), Sp2 (nt -561 to -188 relative to S) and EI/Xp (nt -424 to + 2 relative to HBx) (22) sequences were similarly inserted in pGL3-basic to create pSp1, pSp2 and pXp, respectively. Nucleotides were numbered using first nucleotide of respective ORF as + 1 and the immediate upstream nucleotide as -1. The mutant pCpm was generated by mutating 5′-ATTGG-3′ at nt -21 to -17 of Cp to 5′-AAAAA-3′ using site-directed mutagenesis (Toyobo, China). EII/Cp or EII/Cpm fragments were also inserted downstream of the polyA signal in pSp1, pSp2 and pXp in the same orientation as the upstream promoter to create pSp1-Cp/Cpm, pSp2-Cp/Cpm and pXp-Cp/Cpm, respectively. Schematic presentation of the above reporter plasmids is shown in Figure 1. HMGA1 promoter sequences (nt -4694 to -2695) were inserted into pGL3-basic to create p-4694/-2695 and serial deletion mutants as indicated in Supplementary Figure S16 were then generated using site-directed mutagenesis.

Human HMGA1 (NM_145901) and genotype B HBV (GenBank Accession No. KR232337) HBc, Pol, L/M/SHBs and HBx ORF sequences were cloned upstream of Flag tag in pCMV-C-Flag (pCtrl) (Beyotime, China). Coding sequences of HBe (preC plus amino acid 1–149 of C ORF) from the same genotype B HBV genome were cloned upstream of His tag in pCMV-C-His (Beyotime, China). Recombinant cccDNA plasmid (prcc-

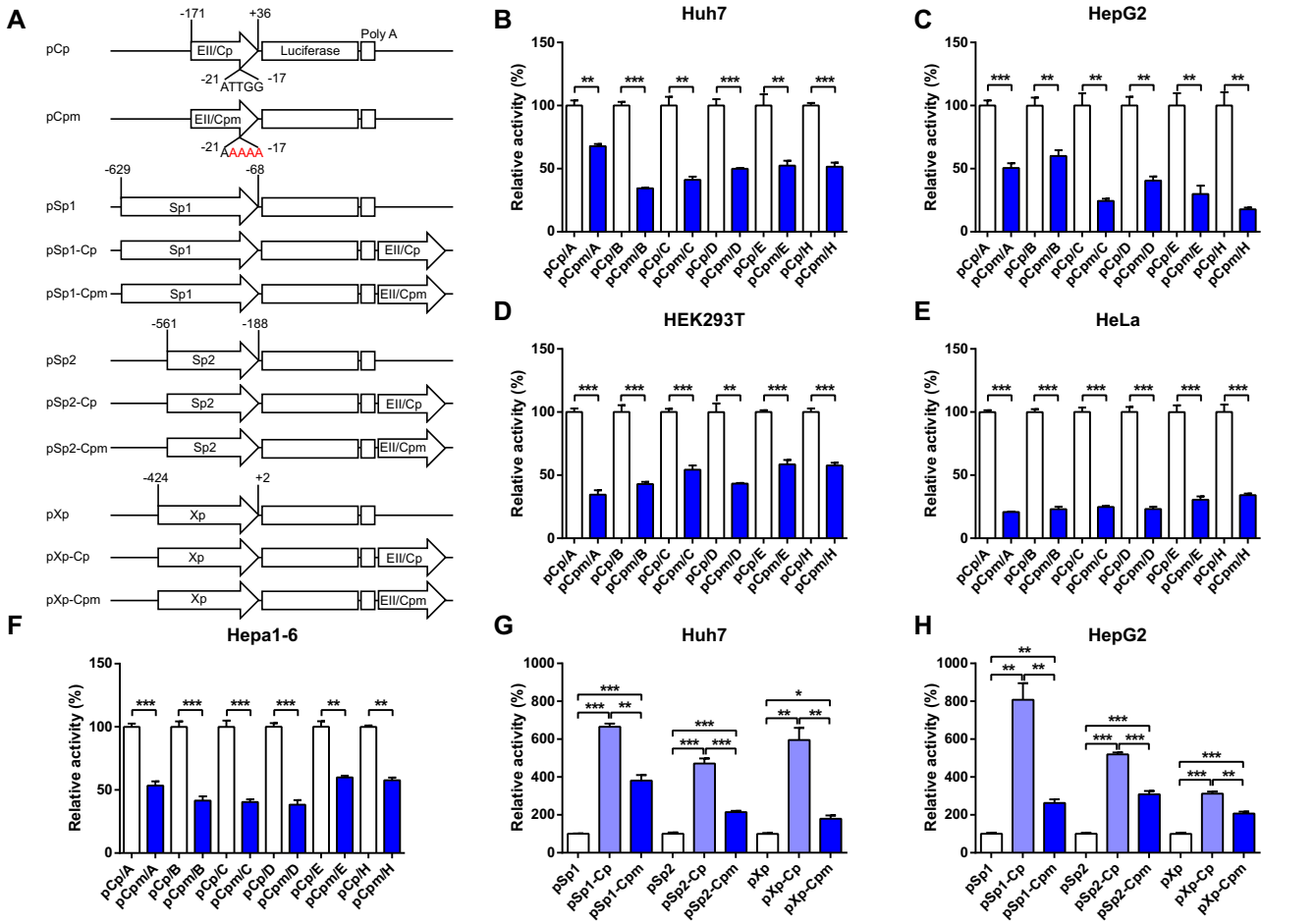

Figure 1. 'ATTGG' element within HBV basal core promoter functions as *cis*-acting positive regulator. (**A**) Schematic representation of HBV enhancer/promoter firefly luciferase reporter plasmids. Numbers denote nucleotide positions relative to the first nucleotide of corresponding ORF. Cpm denotes mutation of 'ATTGG' to 'AAAAA' (red). EII/Cp or EII/Cpm was cloned into Sp1, Sp2 and Xp reporter plasmids downstream of ployA signal in the same direction as upstream promoter to test enhancer activity. Cp or Cpm reporter (0.4 μg) derived from indicated genotypes were co-transfected with 0.1 μg of pRL-TK *Renilla* luciferase reporter into Huh7 (**B**), HepG2 (**C**), HEK293T (**D**), HeLa (**E**) and Hepa1-6 (**F**) cells in 24-well plate. At 48 h post-transfection, cells were lysed and subjected to dual-luciferase reporter assay. The activities of Sp1, Sp2 and Xp in the presence of downstream EII/Cp or EII/Cpm were similarly determined in Huh7 (**G**) and HepG2 (**H**). Group means and SEMs of normalized firefly versus *Renilla* luciferase activity ratios were presented and significances calculated using unpaired two-tailed *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cDNA), Cre recombinase expression plasmid (pCMV-Cre) and HBV replicon plasmid containing a 1.3-copy overlength genotype B genome (p1.3HBV) have been described previously (23,24). X ORF in p1.3HBV and prcccDNA was obliterated by mutating CAA to TAA at 8th amino acid to create p1.3HBV/X^{null} and prcccDNA/X^{null}, respectively. For RNA interference, shRNA coding sequences (Supplementary Table S3) were inserted into pLKO.1 (Addgene, USA).

Transfections were performed using TurboFect (Thermo Fisher, China) and promoter activities were determined using Dual Luciferase Report Assay System (Promega, China), according to the manufacturers' instructions.

Viruses and infections

HBV virions for infection were prepared by concentrating supernatants of HepAD38 cells using Amicon Ultra-15 centrifugal filters (Millipore, China) and quantified using commercial quantitative assay (Adicon, China). HBV infection of HepG2/NTCP and PHH cells was performed at 1000:1 multiplicity of infection (MOI) as previously reported (20,25). Infected cells were washed 12 h later with PBS and changed into fresh media with subsequent media changes every 1–2 days.

For preparation of recombinant lentiviruses, pCDH (System Biosciences) plasmid carrying Flag-tagged HMGA1 gene or pLKO.1-shHMGA1 was co-transfected with helper plasmids psPAX2 and pMD2.G (Addgene, USA) at a ratio of 4:3:1 into HEK293T cells using polyethylenimine (PEI, Sigma, China) as previously described (26). At 6 h post-transfection, culture media were changed and 3 days later virus-containing supernatants were harvested and passed through a 0.45-μm filter and used for transduction of HepG2/NTCP and PHH cells. About 12 h later, infected cells were changed into fresh media.

Recombinant serotype 8 adeno-associated virus (AAV) expressing shRNA targeting murine HMGA1 (AAV-

shHMGA1) and control virus expressing scrambled shRNA (AAV-shCtrl) were purchased from Hanbio technology, China. Recombinant human adenovirus serotype 5 harboring precursor of recombinant cccDNA (Ad/prcccDNA) has been described (23).

In vitro translation

In vitro translated proteins were prepared using TnT T7 Quick Coupled Transcription/Translation System (Promega, China), according to the manufacturer's instruction.

Western blot

Total cell lysates were prepared from cultured cells using SDS lysis buffer (Beyotime, China). Mouse liver tissues were lysed in TBS (10 mM Tris-HCl at pH 7.0, 150 mM NaCl) containing 0.5% NP-40 followed by centrifugation. HBV capsids were first separated by electrophoresis in 1% agarose gel and then blotted onto nitrocellulose for western blot analysis as previously described (27). Nuclear and cytoplasmic fractions were prepared using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China).

Western blot was performed using the following antibodies: anti-Lamin A/C (1:3000, A0249), anti-ANXA1(1:3000, A0249), anti-mouse HMGA1 (1:3000, A4343) from Abclonal; anti-human HMGA1 (1:3000, #7777) and anti-FOXO3α (1:3000, #12829) from Cell Signaling Technology; anti-Flag (1:10000) and anti-β-actin (1:10000) from Sigma; anti-PGC1α (1:3000, 66369–1-lg) and anti-SP1 (1:3000, 21962–1-AP) from Proteintech; anti-HBcAg from DAKO (1:10 000, Copenhagen, Denmark). Mouse anti-HBx mAb 2A7 (1:1000) has been previously reported (28,29).

Immunofluorescence

Cultured cells in 24-well plate were fixed in 4% paraformaldehyde, blocked with 3% BSA in PBST (PBS plus 0.5% Triton X-100), stained with primary antibodies (1:1000) as listed above for western blot for 2 h at room temperature, and finally incubated with Alexa Fluor 488- or 546-labeled secondary antibodies (1:1000, Life Technologies, Waltham, USA) for 1 h at room temperature. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (Sangon, China). Fluorescence microscopy was performed using an AMG EVOS fluorescence microscope (Mill Creek, USA).

DNA-pulldown and co-immunoprecipitation

Streptavidin-coupled Dynabeads (Thermo Fisher, China) and protein A/G agarose (Santa Cruz, China) were used for capturing chemically synthesized biotinylated DNA probes (Genwiz, China) and IgG, respectively. For DNA-pulldown, nuclear fractions from Huh7 cells or *in vitro* translated proteins were mixed with streptavidin-coupled beads and biotinylated DNA probes in binding solution buffer (Beyotime, China). After incubation with rotation at 4°C for 6 h, beads were washed five times with PBST and then mixed in SDS lysis buffer, heated at 100°C for 10 min and analyzed in western blot. Sequences of biotinylated DNA probes are listed in Supplementary Table S4. Co-immunoprecipitation was performed as previously described (30) using antibodies as listed above for western blot.

HBV antigen and nucleic acid analyses

HBsAg, HBeAg and HBsAb were measured using ELISA (Kehua, China). Total cellular RNA was extracted using TRIzol (Invitrogen, China), and 10 μg of RNA was subjected to electrophoresis in 1.5% agarose containing 2.2 mol/l formaldehyde and transferred to positively charged nylon membrane (Roche, Germany). HBV core-associated DNA and nuclear DNA were extracted as previously described (27), and cccDNA-containing extrachromosomal nuclear DNA was prepared using the Hirt method (31). HBV DNA samples were separated in 1% agarose and transferred to nylon membrane. Northern and southern blots were hybridized with DIG-labeled full-length HBV probe and signals were developed using DIG Luminescent Detection Kit (Roche).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using SimpleChIP® Plus Enzymatic Chromatin IP Kit (#9005, Cell Signaling Technology) according to the manufacturer's instructions. Anti-HMGA1 antibodies as listed above for western blot were used.

Realtime PCR for mRNA and HBV cccDNA quantification

For mRNA quantification, total cellular RNA was subjected to reverse transcription using PrimeScript RT reagent Kit (TaKaRa, China) according to the manufacturer's instructions and cDNA was quantified in real-time PCR using SYBR Premix Ex Taq II kit (Takara). PCRs were performed with the following conditions: 95°C/2 min and then 40 cycles of 95°C/10 s, 55°C/15 s and 72°C/20 s on an MXP3000 cycler (Stratagene). For cccDNA quantification, cells were lysed in 0.8 ml lysis buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 150 mM NaCl and 1% SDS) for 20 min at room temperature and then supplemented with 0.2 ml of 2.5M KCl followed by incubated at 4°C overnight with gentle rotation. Lysates were cleared by centrifugation and mixed with 50 μl 20 mg/ml proteinase K. After digestion at 37°C for 6 h, DNA was extracted twice using phenol/chloroform and digested with Plasmid-Safe ATP-dependent DNase (Epicentre Technologies) at 37°C for 4 h. Quantitative real-time PCR was then performed with the following conditions: 95°C/2 min, 40 cycles of 95°C/10 s, 62°C/30 s and 72°C/40 s. Primer sequences use in this work are listed in Supplementary Table S5.

Mice work

For preparation of mice harboring rcccDNA persistence, male C57BL/6 mice transgenic for Cre recombinase (Cre Tg C57BL/6) (23) aged 6–8 weeks were injected with 1.5×10^9 plaque-forming units (PFU) of Ad/prcccDNA

in 200 μl PBS via tail vein. HBV persistence is defined by serum HBsAg positivity at 4 weeks post Ad/prcccDNA injection. For HMGA1 knockdown, 2×10^{11} genome equivalents (geq) of AAV-shHMGA1 or AAV-shCtrl were injected in 200 μl PBS via tail vein. HBV infected PHH cells were collected from human liver chimeric mice infected with HBV as previously described (25). Mouse serum samples were collected at indicated time points through retro-orbital sinus bleeding, and tissues were collected after sacrifice by cervical dislocation. Mouse procedures were approved by the Animal Ethics Committee of School of Basic Medical Sciences, Fudan University. Serum HBV DNA, alanine aminotransferase (ALT), creatine kinase, total bilirubin and creatinine were analyzed using commercial quantitative assays (Adicon, China).

Patient samples and immunohistochemistry analysis

A total of 45 CHB patients at Huashan Hospital of Fudan University were enrolled, with 22 in inactive carrier (IC) phase and 23 in immune tolerant (IT) phase. Clinical information on these patients is listed in Supplementary Table S6. The study received permission (2020–375) from the hospital Ethics Committee and written informed consent was obtained from all enrollees. Total RNA and cccDNA were extracted from liver biopsy samples using TRIzol and Hirt method, respectively, and subjected to RT-qrtPCR and qrtPCR as described above. Intrahepatic expression of HMGA1, HBc and HBx was detected by performing immunohistochemistry on archived liver biopsy FFPE sections using antibodies against HMGA1 (1:1000), HBc (1:500) and HBx (1:500) as listed above for western blot. Relative protein expression levels were expressed using H-SCORE as calculated by Caseviewer 2.0 on images captured by Pannoramic P250 (3DHISTECH, Hungary).

Transcription factor prediction

Potential transcription factor binding sites in promoter sequences were predicted using MatInspector (https://www.genomatix.de/cgi-bin/eldorado/main.pl) (32), employing a cutoff of 80% matrix similarity.

Statistical analysis

Means and standard errors (SEM) from independently repeated experiments were presented and subjected to unpaired two-tailed *t*-test. Group positivity percentages of serum HBV markers were expressed using Kaplan–Meier plot. A P-value < 0.05 was considered statistically significant. GraphPad 5 was used for plotting and statistical tests.

RESULTS

A conserved ATTGG element within HBV EII/Cp positively modulates its activity through HMGA1 binding

Previously, we reported that mutation of an 5′-ATTGG-3′ site (nt -21 to -17) within HBV EII/Cp to 5′-AAAAA-3′ markedly decreased EII/Cp promoter activities in Huh7 and some other human and avian cells (33). Analysis of genome sequences of representative strains from all 10 reported HBV genotypes showed that this site is conserved in all analyzed genomes, and such an ATTGG motif is not found elsewhere within EII/Cp (Supplementary Figure S1). To re-confirm this site's importance for EII/Cp activities, wild type (EII/Cp, nt -171 to + 36) and mutated (EII/Cpm, with ATTGG mutated to AAAAA) EII/Cp sequences derived from common HBV genotypes A-D, E and H were cloned upstream of firefly luciferase reporter (Figure 1A) and analyzed using dual-luciferase reporter assay in human cell lines Huh7, HepG2, HEK293T, HeLa and mouse cell line Hepa1-6. For all tested genotypes in all tested cell lines, EII/Cpm consistently displayed reduced promoter activity compared to EII/Cp (Figure 1B–F). To test whether the ATTGG element has any effect on EII/Cp enhancer activity, genotype B EII/Cp or EII/Cpm was placed downstream of the polyA signal in Sp1-, Sp2- and Xp-driven luciferase reporter plasmids in the same orientation as upstream promoter (Figure 1A). In Huh7 and HepG2 cells, dual-luciferase reporter assay revealed that although both EII/Cp and EII/Cpm enhanced the activities of the respective distal HBV promoters, EII/Cp was considerably more potent than EII/Cpm (Figure 1G and H). Taken together, these results confirmed this highly conserved ATTGG element as a positive regulator of EII/Cp.

The ATTGG site along with neighboring sequences (-75 nt to + 10 nt) were then submitted to MatInspector (32) to search for potential binding sites of known DNA-binding factors. Using a threshold of 80% matrix similarity, possible binding sites for 7 factors from 6 families were identified (Supplementary Table S1). For each of the 7 factors, two different shRNA-expressing plasmids were constructed and verified to knock down endogenous expression in transfected Huh7 cells (Supplementary Figure S2A). EII/Cp and EII/Cpm promoter reporter assays were then performed with shRNA plasmid co-transfection. Among the seven candidates, only knockdown of HMGA1 decreased EII/Cp activity without markedly affecting EII/Cpm activity (Supplementary Figure S2B and C), suggesting that HMGA1 was the most likely factor that acts through the ATTGG site to positively modulate EII/Cp. Similar effects of HMGA1 on promoter activities have been previously demonstrated using similar plasmid-based reporter assays (34,35).

To further probe the interaction between HMGA1 and the ATTGG site, HMGA1 protein level in Huh7 and HepG2 was knocked down using RNA interference or increased using exogenous overexpression, and EII/Cp(m) activities were measured using corresponding luciferase reporters in dual-luciferase assay (Figure 2A and B, Supplementary Figure S3A and B). In both cell lines, HMGA1 protein levels positively correlated with EII/Cp promoter activity, whereas EII/Cpm was hardly affected by changed HMGA1 protein levels (Figure 2A and B, Supplementary Figure S3A and B). Moreover, HMGA1 knockdown or overexpression did not markedly affect the other three HBV promoters (Sp1, Sp2 and EI/Xp) (Supplementary Figure S4). When ATTGG-harboring EII/Cp sequences were placed downstream, however, knockdown of endogenous HMGA1 decreased activity of Sp1, Sp2 and Xp, whereas over-expressed HMGA1 enhanced their activities (Supplementary Figure S4).

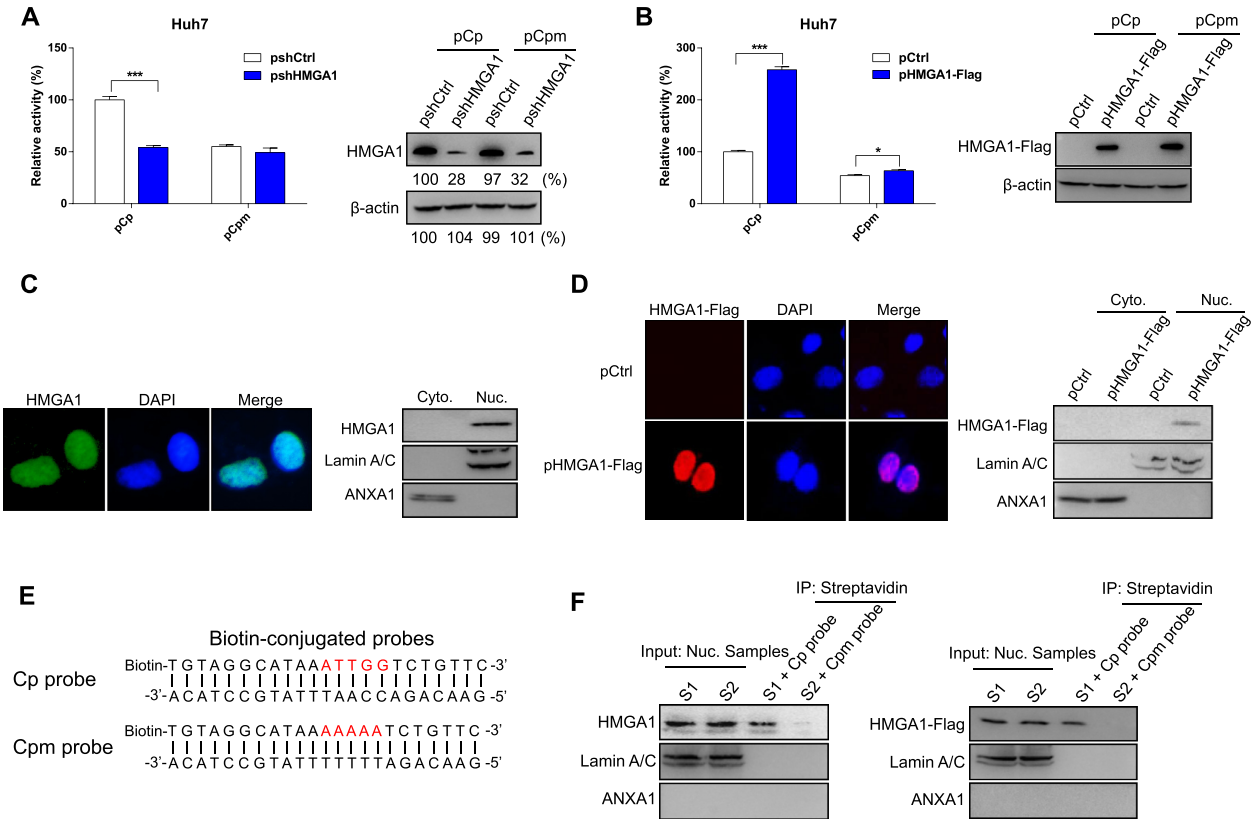

Figure 2. *Cis*-element 'ATTGG' upregulates EII/Cp promoter activity by recruiting HMGA1. Huh7 cells cultured in 24-well plate were co-transfected with 0.4 μg of pCp or pCpm reporter, 0.4 μg of shHMGA1-expressing (pshHMGA1) or control plasmids (pshCtrl) (**A**), or Flag-tagged HMGA1 expression (pHMGA1-Flag) or control plasmids (pCtrl) (**B**), and 0.1 μg of pRL-TK. At 48 h post-transfection, relative promoter activities were measured using dual-luciferase reporter assay. HMGA1 and HMGA1-Flag protein levels were determined in western blot using HMGA1 and Flag antibodies, respectively, and numbers denote normalized densitometry scanning data. Group means and SEMs of normalized firefly versus *Renilla* luciferase activity ratios within transfection group were presented and significances calculated using unpaired two-tailed *t*-test; *, $P < 0.05$; ***, $P < 0.001$. Intracellular distribution of endogenous HMGA1 (**C**) and exogenous HMGA1-Flag (**D**) were analyzed using immunofluorescence and nucleocytoplasmic separation test coupled with western blot. Lamin A/C and annexin A1 (ANXA1) were used as markers of nuclear and cytoplasmic fractions, respectively. (**E**) Biotinylated Cp and Cpm probes (nt -32 to -10 of Cp) used in DNA-pulldown assay. Chemically synthesized probes were added to nuclear protein extracts (S1 and S2) and incubated with streptavidin-coupled beads. After washing, beads were subjected to western blot (**F**).

Immunofluorescence and nuclear/cytoplasmic fractionation assays showed that endogenous as well as exogenous HMAG1 were almost exclusively localized to the nucleus (Figure 2C and D, Supplementary Figure S5A and B). Physical association between HMGA1 and ATTGG-harboring EII/Cp sequences were then tested using Huh7 and HepG2 nuclear extracts in double-stranded DNA probe pull-down assay (Figure 2E). As shown in Figure 2F and Supplementary Figure S5C and D, biotin-labeled wild-type ATTGG-containing EII/Cp probe (nt -32 to -10), but not the mutant EII/Cpm probe, effectively pulled down both endogenously and exogenously expressed HMGA1, as well as *in vitro* translated HMGA1.

Collectively, these data confirmed that the highly conserved ATTGG site, through the binding of HMGA1, functions as a positive *cis*-acting regulatory element of EII/Cp.

HMGA1 upregulates HBV EII/Cp activity through recruiting (co-)transcription factors FOXO3α and PGC1α

As a directly DNA-binding protein, HMGA1 has been shown to activate transcription by recruiting various classical (co-)transcription factors including ATF2, MLXIPL, FOXO family members, PGC1α, MafA, PDX1, SREBF1, CEBPB, TP53 and NF-κB (36). To test whether any of these factors participate in HMGA1-mediated HBV EII/Cp activation, EII/Cp promoter activity reporter assay was performed in Huh7 cells with each one of them knocked down using RNA interference (Supplementary Figure S6A). As shown in Supplementary Figure S6B, knockdown of 5 of the 11 tested factors affected EII/Cp promoter activity: knockdown of FOXO3α and PGC1α resulted in nearly 50–60% reduction (see also Figure 3A), knockdown of CEBPB caused ∼25% reduction, while knockdown of TP53 and NF-κB led to ∼20–25% increase. Due to their more potent effects, FOXO3α and PGC1α were subjected to further characterization. Both immunofluorescence and nuclear/cytoplasmic fractionation assays showed that the majority of cellular FOXO3α was found in the nucleus, whereas PGC1α was almost exclusively localized to the nucleus (Figure 3B and C). In co-immunoprecipitation assay, both endogenous FOXO3α and PGC1α were co-precipitated with endogenous HMGA1 (Figure 3D). Furthermore, biotin-labeled

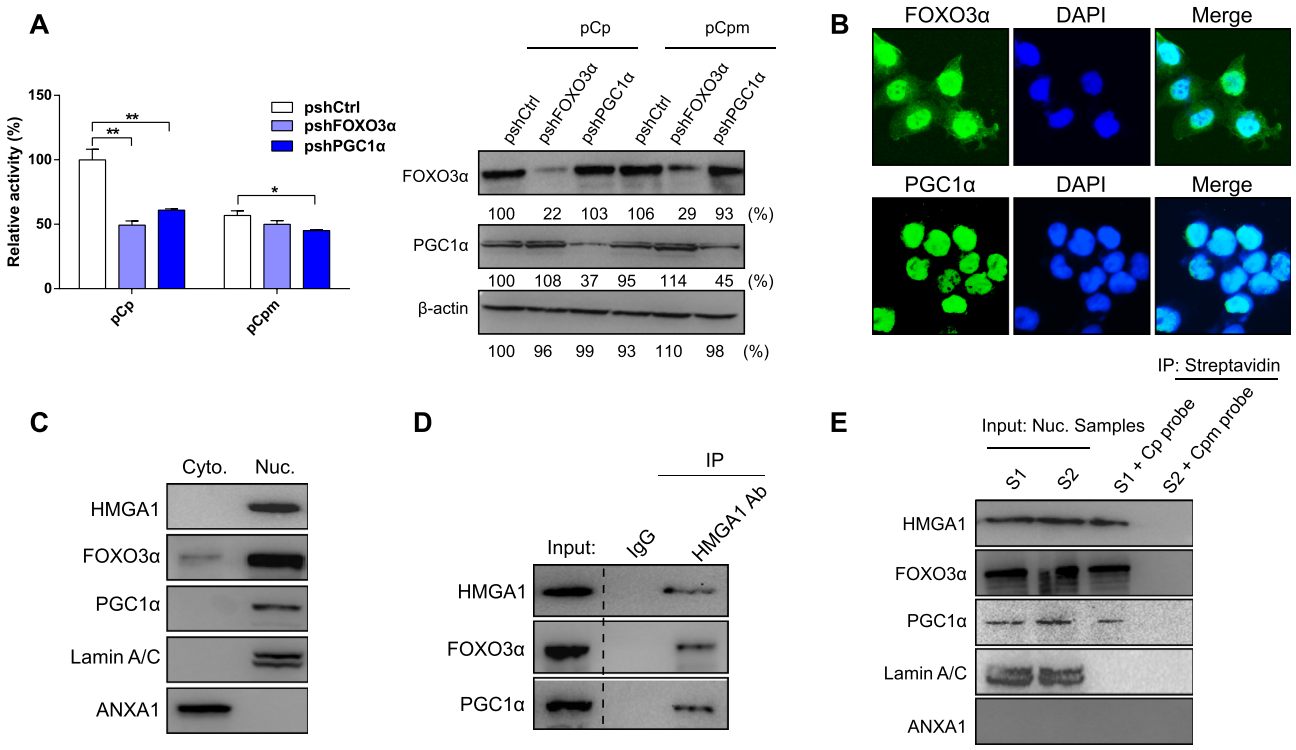

Figure 3. HMGA1 upregulates EII/Cp promoter activity by recruiting FOXO3α and PGC1α to 'ATTGG' element. (**A**) Huh7 cells cultured in 24-well plate were co-transfected with 0.4 μg of pCp or pCpm reporter, 0.4 μg of FOXO3α, PGC1α or control shRNA-expressing plasmids, and 0.1 μg of pRL-TK. At 48 h post-transfection, relative promoter activities were measured using dual-luciferase reporter assay. FOXO3α and PGC1α protein levels were determined in western blot and numbers denote normalized densitometry scanning data. Group means and SEMs of normalized firefly versus *Renilla* luciferase activity ratios within transfection group were presented and significances calculated using unpaired two-tailed *t*-test; *, $P < 0.05$; **, $P < 0.01$. Intracellular distribution of endogenous FOXO3α and PGC1α were analyzed using immunofluorescence (**B**) and nucleocytoplasmic separation test coupled with western blot (**C**). Lamin A/C and annexin A1 (ANXA1) were used as markers of nuclear and cytoplasmic fractions, respectively. (**D**) Co-immunoprecipitation of endogenous FOXO3α and PGC1α with endogenous HMGA1. (**E**) Endogenous FOXO3α and PGC1α, in addition to HMGA1, were pulled down by biotinylated Cp and Cpm probes (nt -32 to -10 of Cp) as shown in Figure 2E. Chemically synthesized probes were added to nuclear protein extracts (S1 and S2) and incubated with streptavidin-coupled beads. After washing, beads were subjected to western blot.

wild-type ATTGG-containing EII/Cp probe (nt -32 to -10), but not the mutant EII/Cpm probe, effectively pulled down both endogenous FOXO3α and PGC1α, in addition to HMGA1 (Figures 2E, F and 3E). More importantly, *in vitro* translated FOXO3α and PGC1α were captured by biotin-labeled EII/Cp probe, but not EII/Cpm probe, and only in the presence of HMGA1 (Supplementary Figure S5D). Such recruitment of FOXO3α and PGC1α by HMGA1 to ATTGG site might mechanistically contribute toward their participation in HMGA1-mediated upregulation of EII/Cp (Figure 3A). Indeed, when endogenous HMGA1 was knocked down through RNA interference, further knockdown of either FOXO3α, PGC1α or both only marginally affected EII/Cp promoter activity (Supplementary Figure S7).

HMGA1-mediated activation via the ATTGG element enhances HBV gene expression and genome replication

Since HMGA1 binding to ATTGG element positively modulates EII/Cp, and potentially also the other 3 HBV promoters via elevated enhancer activity (Figure 1), the effects of HMGA1 on viral gene expression and genome replication were explored using a previously established recombinant cccDNA (rcccDNA) system (23). In this system, rcccDNA is produced intracellularly from transfected precursor rcccDNA (prcccDNA) plasmid by the action of Cre recombinase. The produced rcccDNA is highly similar to wild type cccDNA, except an artificially inserted small intron that is removed post-transcriptionally through splicing.

In Huh7 cells transfected with prcccDNA and Cre-expressing plasmids, knockdown of endogenous HMGA1 markedly decreased the levels of cellular HBV 3.5 kb and 2.4/2.1 kb RNA (Figure 4A), indicating down-regulated viral transcription, as well as levels of capsid-associated viral DNA replication intermediates in the cytoplasm, indicating reduced viral replication (Figure 4A). Moderate reductions in secreted HBsAg and HBeAg levels were also observed (Figure 4A). In contrast, level of rcccDNA, which was produced from transfected prcccDNA and thus independently of HBV enhancer/promoters, was not affected (Figure 4A). Conversely, when Huh7 cells were supplemented with exogenous HMGA1, elevated HBV RNA transcription and genome replication were observed, with moderate increases of secreted antigens and no significant changes in rcccDNA level (Figure 4B). Furthermore, such promoting effects of over-expressed HMGA1 were dose-dependent

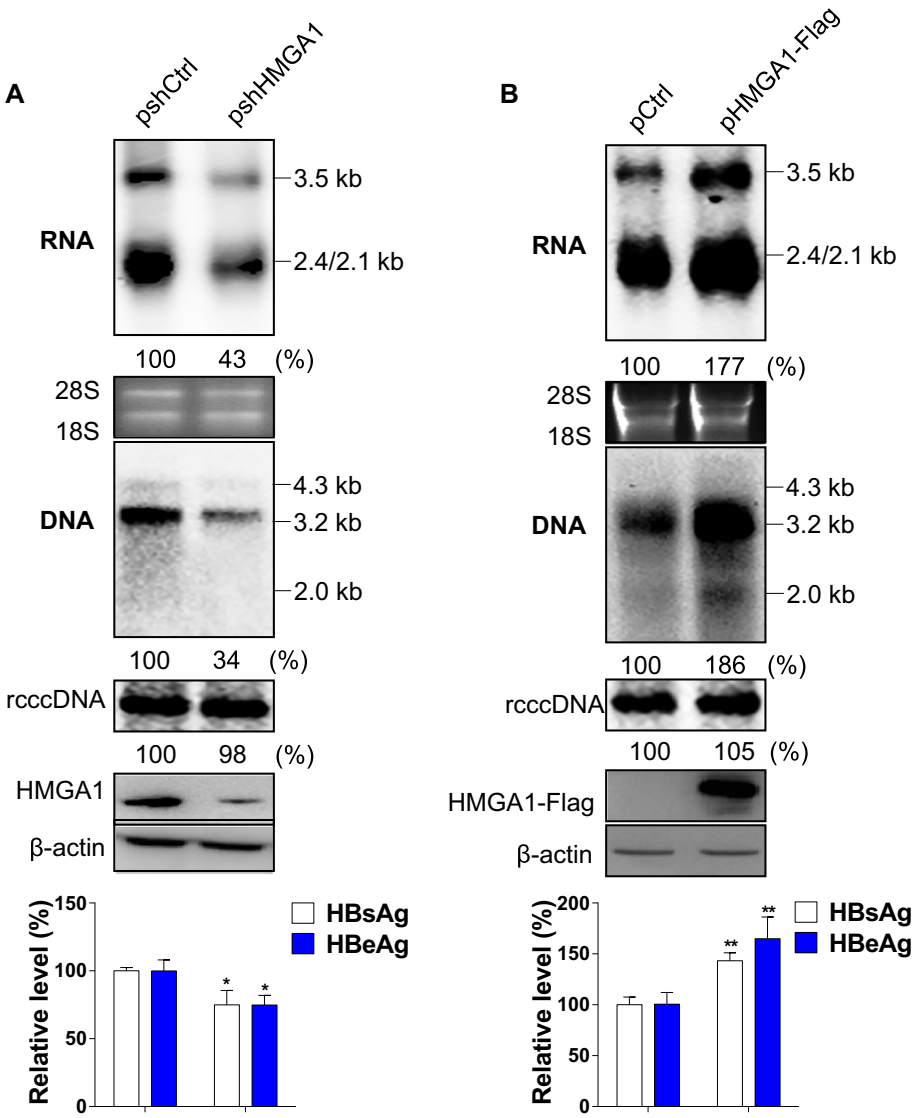

Figure 4. HMGA1 promotes HBV gene expression and genome replication in Huh7 cells. Huh7 cells in six-well plates were transfected with 1 μg of prcccDNA and 1 μg of pCMV-Cre, plus 1 μg of pshHMGA1 or pshCtrl (**A**) or pHMGA1-Flag or pCtrl (**B**). At day 3 post transfection, intracellular HBV RNA, core-associated DNA and rcccDNA were assayed by northern and southern blot, respectively. Endogenous and exogenous HMGA1 protein levels were determined in western blot using HMGA1 and Flag antibodies, respectively. HBsAg and HBeAg levels in culture media were measured by ELISA. The relative levels of HBV RNA and DNA were quantified using densitometry scanning. Group means and SEMs of normalized antigen levels were presented. Significances were calculated using unpaired two-tailed *t*-test; *, $P < 0.05$; **, $P < 0.01$.

(Supplementary Figure S8). On the other hand, replication capacity of mutant prcccDNA, wherein the HMGA1 responsive element ATTGG within EII/Cp was mutated to non-HMGA1-binding AAAAA, was not only markedly reduced compared to wild-type prcccDNA but also was not affected by HMGA1 overexpression (Supplementary Figure S9). Chromatin immunoprecipitation (ChIP) assay then confirmed occupancy of HMGA1 on wild-type rcccDNA but not ATTGG-to-AAAAA mutant rcccDNA (Supplementary Figure S10). Finally, knockdown of endogenous FOXO3α and PGC1α displayed similar but comparatively lower inhibitory effects on HBV replication and antigen production (Supplementary Figure S11), and such effects were further reduced when endogenous HMGA1 was simultaneously knocked down (Supplementary Figure S12). These results collectively showed that, consistent with its transcription-activating effects on HBV enhancers and promoters through the ATTGG element (Figure 1), HMGA1 indeed acts to promote HBV gene expression and genome replication.

Next, effects of HMGA1-mediated regulation on wild-type cccDNA produced during HBV infection were assessed using *in vitro* infection systems. Endogenous HMGA1 was knocked down in HepG2/NTCP cells using shRNA-expressing recombinant lentivirus 3 days before infection by HBV (Supplementary Figure S13A). Serial measurement of HBV antigens secreted by infected cells showed that after HBV antigens brought in the inoculates had been washed off by 2 days post HBV infection (d.p.i.), control HepG2/NTCP cells displayed the expected grad-

ual rise of extracellular HBeAg and HBsAg (Supplementary Figure S13B and C). In contrast, cells with HMGA1 knocked down only produced low but detectable HBeAg, and barely detectable HBsAg by 7 d.p.i. (Supplementary Figure S13B and C). Similarly marked repression caused by HMGA1 knockdown was also observed when intracellular HBV RNA, capsid-associated DNA replication intermediates and nuclear cccDNA were analyzed (Supplementary Figure S13D).

Since it could not be ruled out that HMGA1 might also affect HBV life cycle steps preceding cccDNA generation, which would result in differences in cccDNA levels and complicate attribution of the effects observed in Supplementary Figure S13, we infected HepG2/NTCP cells first with HBV, allowed 2 days for cccDNA to be produced and viral transcription to begin (Supplementary Figure S13A) and then infected HBV-infected cells with HMGA1 shRNA-expressing or HMGA1 overexpression lentiviruses (Figure 5A). As shown in Figure 5B and C, a positive correlation between HMGA1 levels and HBV RNA transcription, antigen production and genome replication was again observed. Furthermore, similar results were reproduced using primary human hepatocytes (PHH) as target cells (Supplementary Figure S14). In both HBV-infected HepG2/NTCP and PHH cells, ChIP assay confirmed occupancy of HMGA1 on cccDNA (Supplementary Figure S15). Clearly, high cellular HMGA1 is favorable for efficient gene expression and genome replication during HBV infection, which most likely is mediated through its binding to cccDNA and recruitment of transcription factors such as FOXO3α and PGC1α.

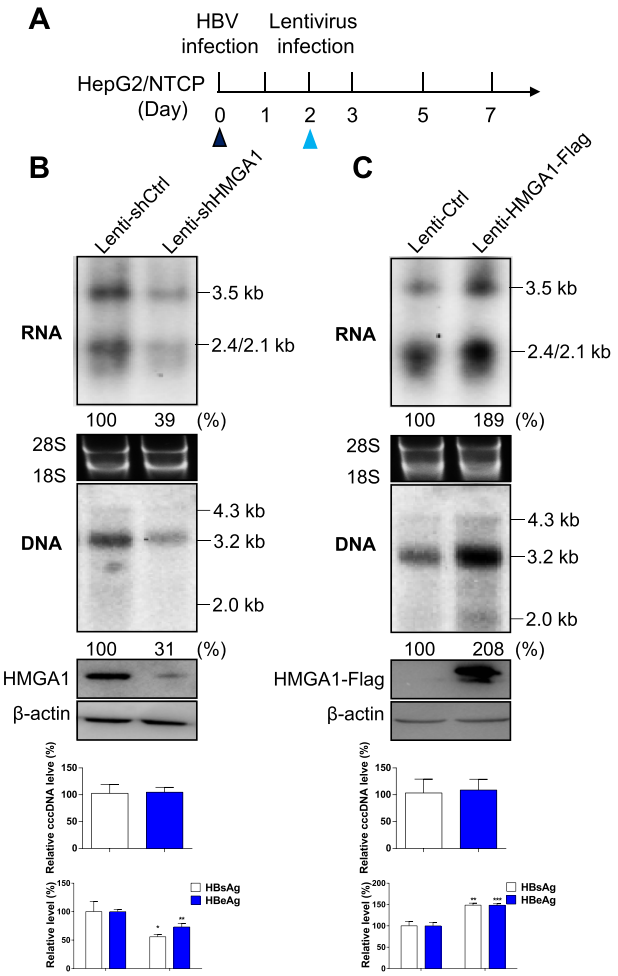

Figure 5. HMGA1 promotes HBV gene expression and replication in HBV-infected HepG2/NTCP cells. HepG2/NTCP cells were infected with HBV virions at a MOI of 1000 geq/cell overnight. Culture media were changed the next day, and at day 2, cells were transduced by shHMGA1-expressing lentivirus or control virus (Lenti-shHMAG1 or Lenti-shCtrl) (**A** and **B**), or Lenti-HMGA1-Flag or control virus Lenti-Ctrl (**A** and **C**). Culture media were changed at day 3, and then every 2 days. At day 7, intracellular HBV RNA, core-associated DNA and cccDNA were examined using northern and southern blot, and qrtPCR, respectively. Endogenous and exogenous HMGA1 were determined in western blot using anti-HMGA1 and anti-Flag antibodies, respectively. HBsAg and HBeAg in culture media were assayed using ELISA. The relative levels of HBV RNA and DNA were quantified using densitometry scanning. Group means and SEMs of normalized values within transfection group were presented. Data of Lenti-shHMGA1or Lenti-HMGA1-Flag-transduced groups were compared against control virus group using unpaired two-tailed *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

HBV upregulates endogenous HMGA1 expression through X protein

During the evaluation of HMGA1's effects on HBV in HepG2/NTCP and PHH cells, it was surprisingly observed that HBV-infected cells displayed elevated endogenous HMGA1 protein expression compared to uninfected cells (Figure 6A). Similar observation was also made in prcccDNA/Cre-transfected Huh7 cells (Supplementary Figure S16A), and Huh7 cells transfected with 1.3-fold terminally redundant HBV genome (p1.3HBV, Supplementary Figure S16B). To probe viral component(s) responsible for inducing HMGA1 upregulation, HBV-encoded proteins were individually overexpressed in Huh7 cells and only X protein (HBx) increased endogenous HMGA1 expression (Supplementary Figure S16B). On the other hand, mutant p1.3HBV and prcccDNA with HBx ORF obliterated failed to upregulate HMGA1 (Figure 6B and Supplementary Figure S16A). Expectedly, co-transfection with HBx expression plasmid increased 3.5 kb RNA synthesis and genome replication by prcccDNA/X^{null} with concurrent upregulation of endogenous HMGA1 expression (Supplementary Figure S17). These data demonstrated that HBV, through the expression of HBx, enhances expression of endogenous HMGA1.

Mechanisms of HBx-induced HMGA1 upregulation were then explored. As shown in Figure 6C, transfection with either p1.3HBV or HBx overexpression plasmid increased HMGA1 mRNA level and promoter assay confirmed that in both cases, HMGA1 promoter (nt -4694 to -2695, as previously characterized by other groups (37,38)) activity was prominently increased (Figure 6D), indicating HBx-mediated transcriptional activation of HMGA1 promoter. To identify HBx-responsive element(s) in HMGA1 promoter, serial deletion mutants were generated and tested in promoter activity assay with or without HBx overexpression. As shown in Supplementary Figure S16C, HBx-mediated activation was only observed when the segment between nt -3444 and nt -3195 (250 bp) was present.

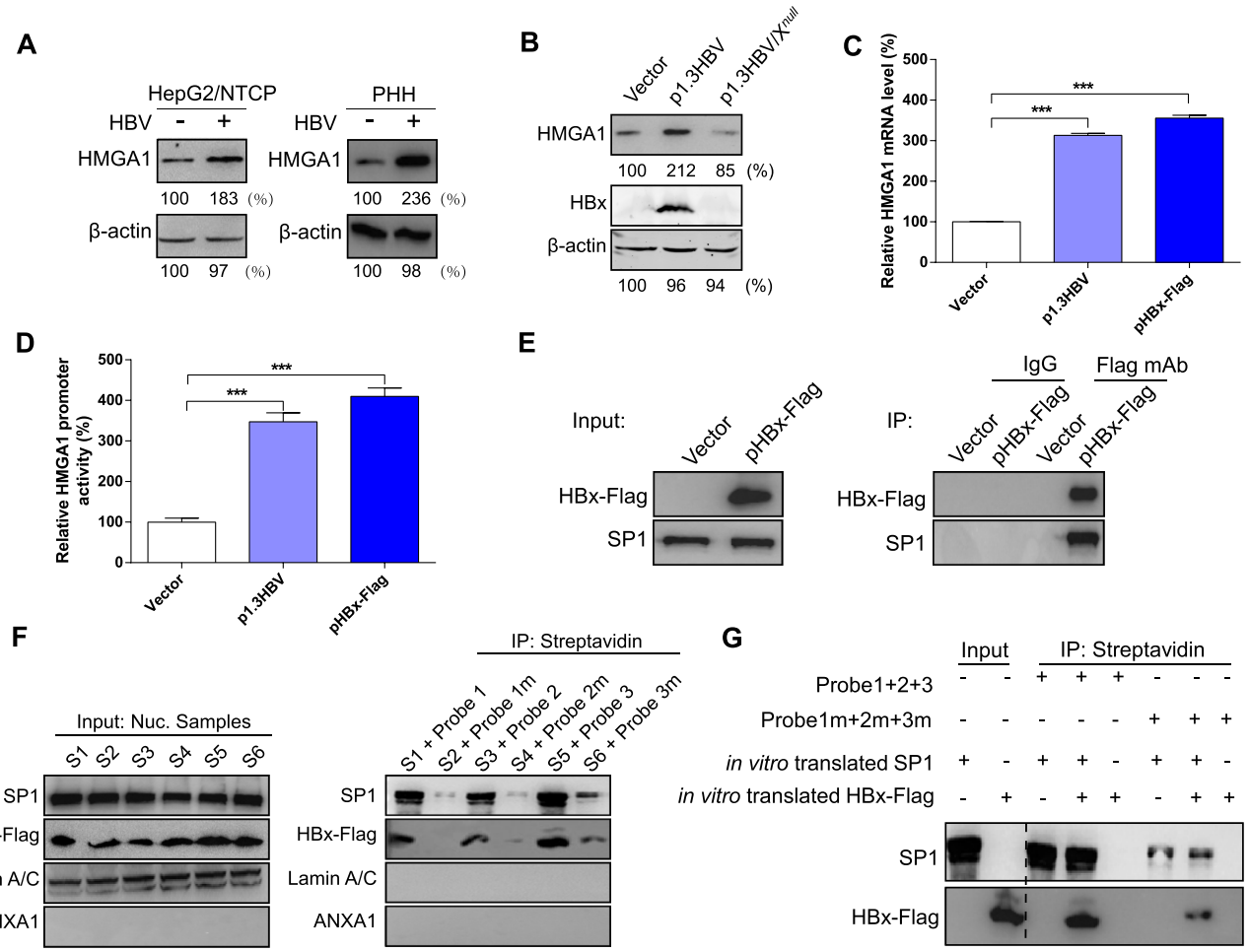

Figure 6. HBV upregulates HMGA1 expression via HBx. (**A**) Endogenous HMGA1 in HepG2/NTCP and primary human hepatocytes (PHH) cells with or without HBV infection were analyzed in western blot. (**B**) Huh7 cells were transfected with HBV replicon plasmid p1.3HBV, or p1.3HBV with HBx obliterated (p1.3HBV/X^{null}), or control vector. At day 2 post transfection, intracellular HMGA1 was analyzed in western blot. (**C**) Huh7 cells were transfected with p1.3HBV, Flag-tagged HBx expression plasmid pHBx-Flag or control vector. At day 2 post transfection, HMGA1 mRNA levels were measured using RT-qrtPCR. (**D**) HMGA1 promoter reporter plasmid and pRL-TK were co-transfected with control vector, p1.3HBV or pHBx-Flag into Huh7 cells. At day 2 post transfection, cells were lysed and promoter activities were measured using dual-luciferase reporter assay. (**E**) Huh7 cells transfected with pHBx-Flag or control vector were subjected to co-immunoprecipitation analysis using anti-Flag antibody. (**F**) Endogenous SP1 and exogenous Flag-tagged HBx were pulled down by biotinylated probes encompassing the three SP1 binding sites in HMGA1 promoter (Probes 1–3) or corresponding mutant probes with SP1 binding sites mutated (Probes 1m-3m) as shown in Supplementary Table S4. Chemically synthesized probes were added to nuclear protein extracts (S1-6) and incubated with streptavidin-coupled beads. (**G**) *In vitro* translated SP1 and HBx-Flag were incubated with a mixture of biotinylated wild-type or mutant SP1 binding site probes and streptavidin-coupled beads. After washing, beads were subjected to western blot. Numbers denote normalized densitometry scanning data (A and B). Group means and SEMs of normalized values within transfection group were presented and significances calculated using unpaired two-tailed *t*-test; ***, $P < 0.001$.

Since HBx generally does not bind DNA, interaction between HBx and this segment in HMGA1 promoter most likely involves other DNA-binding host factor(s). This HBx-responsive segment has a GC content of 89.2% and previous studies have identified three GC box-containing binding sites for Sp1 transcription factor (SP1) within this region using MatInspecter (Supplementary Table S2) (37,38). Promoter activity assay showed that SP1 overexpression indeed activated HMGA1 promoter, and SP1-responsive element was mapped to the same segment between nt -3444 and -3195 (Supplementary Figure S16C). In both immunofluorescence and nuclear/cytoplasmic fractionation assays, HBx expressed from transfected plasmid in Huh7 cells was identified in both cytoplasm and the nucleus, whereas endogenous SP1 was almost exclusively localized to the nucleus (Supplementary Figure S16D). Co-immunoprecipitation assay then confirmed an association between HBx and SP1 (Figure 6E), while DNA-pulldown assay showed that all three SP1-binding sites within HBx-responsive element of HMGA1 promoter were capable of capturing SP1 and HBx in nuclear extracts (Figure 6F). More importantly, *in vitro* translated HBx was specifically captured by a mixture of the three biotin-labeled SP1 binding site probes, but only in the presence of *in vitro* translated SP1 (Figure 6G). Finally, HMGA1 promoter with the three SP1-binding sites mutated displayed markedly reduced activity and responsiveness to HBx activation (Supplementary Figure S16E). These results

demonstrated that HBx-mediated activation of HMGA1 most likely involves its recruitment to HMGA1 promoter by SP1.

Correlation of intrahepatic HMGA1 expression with HBV activity in immunotolerant phase patients and inactive carriers

The reciprocal enhancement between HBV and endogenous HMGA1 suggests the existence of a positive feedback loop, which would entail a positive correlation between HMGA1 and HBV transcription, as well as downstream antigen production and viral replication, in naturally infected hepatocytes *in vivo*. To test this possibility, chronically HBV-infected patients in immunotolerant phase ($n = 23$), defined by persistent viral activity without ongoing inflammation, and inactive carriers ($n = 22$), characterized by largely suppressed viral activity in the absence of active inflammation, were analyzed. Clinical profiles showed significantly higher serum viral loads and HBsAg in immunotolerant phase patients compared to inactive carriers (Supplementary Table S6). Liver biopsies were performed and samples were subjected to RNA analysis using quantitative real time PCR and protein analysis using immunohistochemistry. As shown in Figure 7A and B, immunotolerant phase patients displayed significantly higher intrahepatic HBV 3.5kb RNA (normalized using intrahepatic cccDNA levels) and HMGA1 mRNA (normalized using actin mRNA) levels compared to inactive carriers. Immunohistochemistry also confirmed higher HMGA1, and slightly higher HBc and HBx protein expression in immunotolerant phase patients (Figure 7C–F). Collectively, these data suggest that there is indeed a positive correlation between HMGA1 and HBV activity in infected patients.

HMGA1-targeting RNA-interference therapy induced clearance of HBV persistence in mice

To further explore the importance of HMGA1 for HBV *in vivo*, endogenous HMGA1 was targeted in the mouse model of HBV persistence based on prcccDNA/Cre system (23). Recombinant adeno-associated virus expressing HMGA1-targeing shRNA (AAV-shHMGA1) was constructed and demonstrated to knockdown intrahepatic endogenous HMGA1 expression in mice for up to 10 weeks after a single injection through tail vein (Supplementary Figure S18). Cre-transgenic C57BL/6 mice were first injected with recombinant adenovirus carrying precursor cccDNA (Ad/prcccDNA) through tail vein, and 4 weeks post injection (w.p.i.), mice with persistently positive serum HBsAg were taken as harboring HBV persistence and injected with AAV-shHMGA1 or control virus (AAV-shCtrl, Figure 8A). Regular serum sampling up to 10 w.p.i. showed that AAV-shHMGA1 caused significantly quicker decrease of serum HBsAg, HBeAg and HBV DNA compared to AAV-shCtrl, and by the end of 10 weeks, 2 out of 6 AAV-shHMGA1-injected mice, but none of 6 AAV-Ctrl-injected mice, had cleared all three serum HBV markers (Figure 8B–E). Regardless of outcome, no serum HBsAb development was observed in either group (Figure 8C), while ALT, creatine kinase, total bilirubin and creatinine remained generally normal in both groups (Figure 8F–I). Mice were sacrificed at 10 w.p.i. and liver samples were taken for analysis of intracellular HBV RNA and DNA replication intermediates as well as core protein. As shown in Figure 8J, these markers were at prominently lower levels in AAV-shHMGA1-injected mice, and more importantly, they were undetectable in the two AAV-shHMGA1-injected mice that had achieved clearance of serum HBV markers.

The experiment was then repeated with longer follow-up of 20 weeks. At 20 w.p.i., all 5 out of 5 AAV-shHMGA1-injected rcccDNA persistence mice had achieved clearance of both serum and intrahepatic markers, including intrahepatic rcccDNA, while only 1 out of 5 AAV-shCtrl-injected mice displayed markedly lower or near cut-off levels of HBV markers compared to other mice in the same group (Supplementary Figure S19). Only one AAV-shHMGA1-injected mice developed detectable serum HBsAb. Failure to detect intrahepatic rcccDNA in AAV-shHMGA1-injected mice indicated that HMGA1-targeting resulted in true clearance, instead of temporary repression, of rcccDNA in this model (Supplementary Figure S19). These results not only corroborated data obtained *in vitro* and illustrated the importance of HMGA1 for HBV gene expression and viral replication but also support HMGA1 as a potential therapeutic target for the treatment of chronic HBV infection.

DISCUSSION

HBV cccDNA is highly stable and no intrinsic intracellular process is known to be capable of degrading cccDNA. Even in self-limiting acute infections that have resolved and in chronic infections that have reached treatment-induced functional cure, cccDNA could persist in a transcriptionally inactive state and pose the risk of reactivation as well as development of HCC (39,40). When cccDNA is being transcribed, transcription levels can vary significantly, as exemplified by the higher intrahepatic HBV RNA in immune tolerant phase patients compared to inactive CHB patients (Figure 7A), which also translates to higher serum HBsAg and HBV DNA in the former (Supplementary Table S6). Since the identification of basal core promoter (41) and the partially overlapping enhancer II (42), multiple host factors have been reported to regulate this key *cis*-regulatory element responsible for pgRNA transcription (43). The ATTGG site at nt -21 to -17, as well as the neighboring nucleotide sequences, is highly conserved among most HBV genotypes (Supplementary Figure S1), suggesting viral dependence on regulation effected through this region, including HMGA1-mediated transcriptional activation reported in this work. Despite the overlapping between EII and Cp, enhancer activity has not been attributed to Cp in the past. Results presented here, however, indicate that HMGA1 binding to ATTGG site, which is not located within EII as classically defined, could also enhance transcription from distal promoters (Supplementary Figure S4). Although mutating ATTGG to AAAAA obliterates HMGA1 binding and activation (Figure 2), it could not be ruled out that other neighboring nucleotides may also participate in HMGA1-binding and subsequent recruitment of other factors.

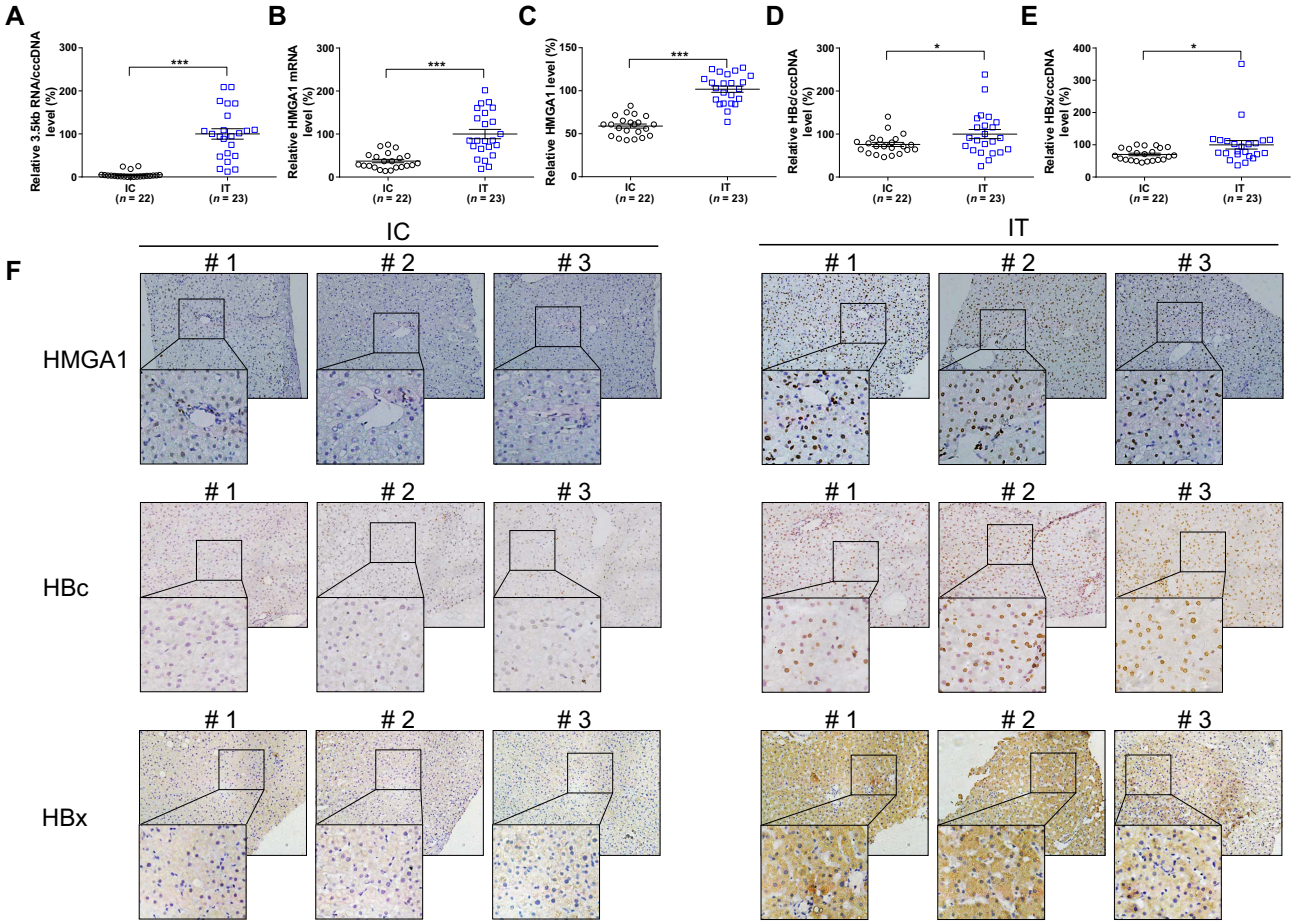

Figure 7. Correlation between intrahepatic HMGA1 level and HBV activity in immune tolerant and inactive carrier phase CHB patients. Liver biopsy samples from inactive carrier ($n = 22$) and immune tolerant ($n = 23$) phase CHB patients were used for qrtPCR analysis of HBV 3.5 kb RNA (**A**), as well as and HMGA1 mRNA (**B**). HBV cccDNA and endogenous actin mRNA levels were also quantitated and used for normalization, respectively. Immunostaining of HMGA1 (**C**), HBc (**D**) and HBx (**E**) proteins in addition to H&E staining was also performed, and representative images are presented (**F**). Protein levels were evaluated using H-SCORE analysis. HBc and HBx protein levels were normalized using cccDNA levels. Group means and SEMs were presented and significances calculated using unpaired two-tailed *t*-test. **, $P < 0.01$; ***, $P < 0.001$.

Involvement of FOXO3α and PGC1α in HMGA1-mediated activation is supported by functional analysis (Figure 3A, Supplementary Figure S7 and S12) and, more importantly, their HMGA1-dependent association with the ATTGG-containing segment of EII/Cp (Figure 3E and Supplementary Figure S5D). FOXO3α is a ubiquitously expressed DNA-binding factor that can act both as a pioneer factor that opens chromatin to facilitate binding by other chromatin remodelers and transcription factors, and as a classical transcription factor that regulates transcriptional activation through interaction with and recruitment of other chromatin remodelers and transcription factors (44). FOXO3α has not been previously implicated in regulation of HBV transcription and no canonical binding site for FOXO3α is identified within EII/Cp probe used in this work. In light of its dependence on HMGA1 for association with EII/Cp probe (Supplementary Figure S5D), FOXO3α most likely activates EII/Cp as a classical transcription factor in this context. In contrast, PGC1α is a classical co-transcription factor that is recruited by multiple directly DNA-binding nuclear receptors and modulates transcriptional activity by interacting with other transcription machinery components (45). Previous studies have linked PGC1α and retinoid X receptor alpha, farnesoid X receptor alpha, liver receptor homolog 1 and estrogen-related receptors alpha and beta (46,47) to activation of HBV transcription and replication, yet convincing evidence of its association with EII/Cp has been scarce (48). No canonical binding sites for these nuclear receptors can be identified within our EII/Cp probe either. HMGA1-dependent association of PGC1α with EII/Cp probe (Supplementary Figure S5D) therefore indicates its involvement in EII/Cp activation through HMGA1 recruitment (Figure 3). Possible additional factor(s) involved in recruiting PGC1α to HMGA1-bound EII/Cp remain(s) to be elucidated. Furthermore, CEBPB, TP53 and NF-κB, which had also been previously linked to regulation of HBV transcription (6,43), were shown to affect EII/Cp activity in a HMGA1-related manner here (Supplementary Figure S6). Whether these well-known transcription regulators directly act on HMGA1-bound EII/Cp, or indirectly through other mechanisms, are not yet clear. Taken together, these

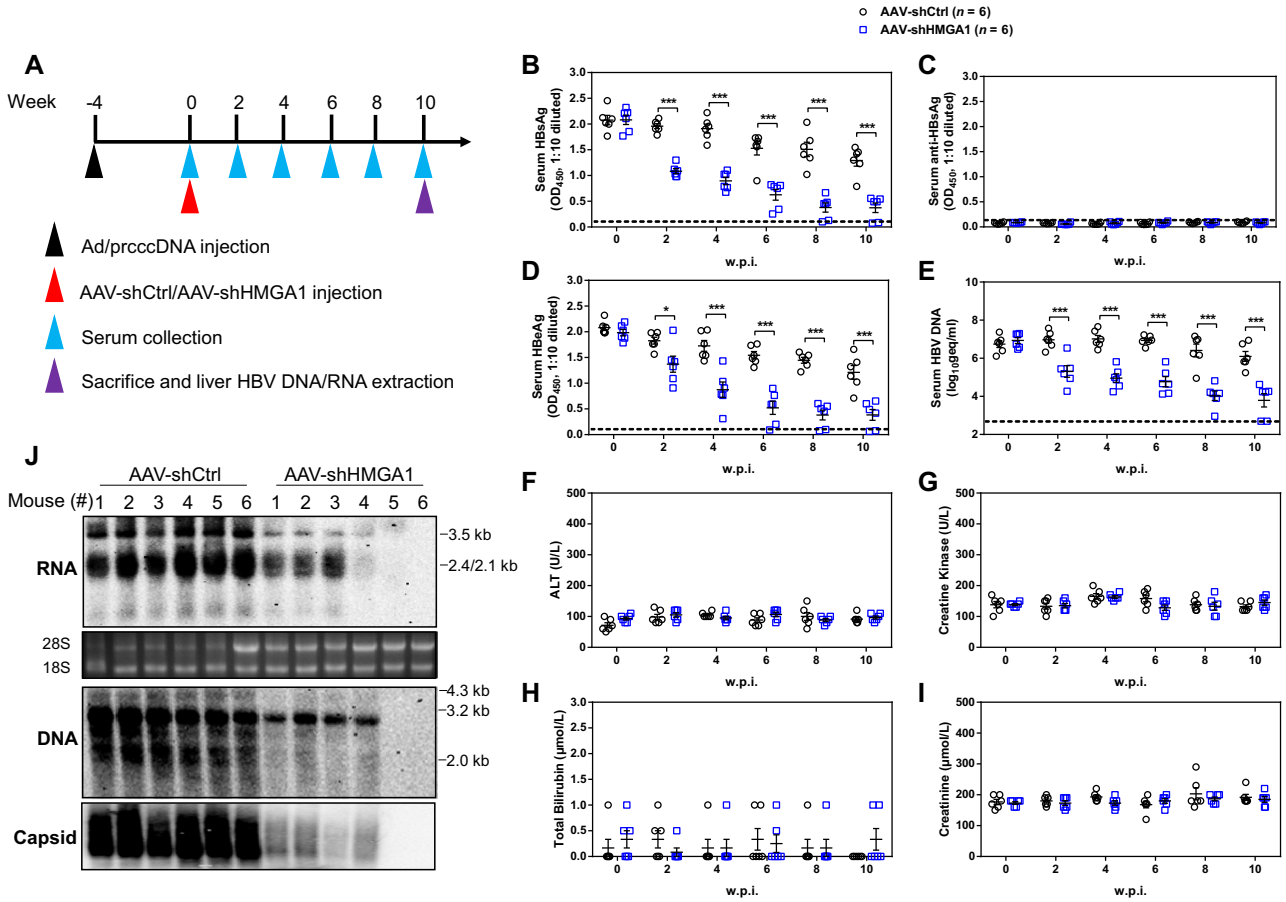

Figure 8. Effects of HMGA1 knockdown in prcccDNA-based mouse model of HBV persistence. (**A**) C57BL/6 mice transgenic for Cre recombinase were injected with Ad/prcccDNA and mice that remained positive for serum HBV antigen for 4 weeks after injection were injected with 2×10^{11} geq of AAV-shHMGA1 ($n = 6$) or AAV-shCtrl ($n = 6$). Sera were collected at indicated time points. At 10 weeks post injection (w.p.i.) of AAV, mice were sacrificed and liver samples taken. Serum HBsAg (**B**), HBsAb (**C**) and HBeAg (**D**) were assayed using ELISA. Serum HBV DNA (**E**), ALT (**F**), creatine kinase (**G**), total bilirubin (**H**) and creatinine (**I**) were measured using commercial quantitative assays. Statistical significances are calculated using unpaired two-tailed *t*-test. *, $P < 0.05$; ***, $P < 0.001$. (**J**) Intrahepatic HBV RNA and capsid-associated HBV DNA were analyzed using northern and southern blot, respectively, and core proteins analyzed using native agarose gel electrophoresis followed by western blot.

data demonstrate that HMGA1-binding enables recruitment of other transcription (co-)factors to achieve EII/Cp activation.

HBx has long been accepted as a key activator of cccDNA transcription that acts through diverse direct and indirect mechanisms (8), especially during the initial intracellular phase of infection (49). HBx-mediated upregulation of HMGA1 expression (Figure 6) is consistent with such an established role of HBx and reiterates its importance for HBV life cycle. The reciprocal promotion between HBV and HMGA1 constitutes a positive feedback loop that could result in a positive correlation between levels of HBV activities and HMGA1 protein, as demonstrated in immune tolerant and inactive carrier CHB patients (Figure 7). Since HBx-related and unrelated regulation of HBV involve many other host factors acting simultaneously, to which extent HMGA1 affect HBV activity *in vivo* remains to be clarified. Further complications could be expected when host immune responses come into play in immune active and reactivation CHB patients. How the HBV-HMGA1 equilibrium transitions from the high-high state in IT phase to the low-low state in IC phase is also an important question that warrants attention.

Despite HMGA1's important roles in development and carcinogenesis, study of its expressional regulation has been relatively limited. Results presented here are consistent with previous reports linking SP1 binding sites in HMGA1 promoter to both basal and inducible transcription (37,38). Transcriptional regulation by HBx via association with SP1 has also been reported for host protein and non-coding RNA genes (50,51), and might thus constitute a common mechanism of HBx-mediated transcriptional modulation.

Role of HMGA1 in HCC has not been extensively studied compared to many other common tumor types. Recently, increase in HMGA1 mRNA and protein expression was correlated with development from normal liver to cirrhosis and HCC: nearly half of tested HCC samples expressed HMGA1, which was associated with earlier progression and worse outcome (52). Given the long-established link between HBx and HCC, it is tempting to speculate on possible involvement of HBx-mediated HMGA1 upregulation in HBV-related HCC development.

Since IT phase usually precedes IC phase, whether continued higher HMGA1 expression during IT phase contributes in any way toward HCC development later in life is an important question that nevertheless would prove difficult to answer. HMGA1 levels in reactivation phase, which is temporally closest to HCC development, could be examined as done in this work, yet fluctuations in viral activities and host inflammation responses would undoubtedly complicate data analysis.

Results obtained using mice harboring rcccDNA-based HBV persistence that were subjected to HMGA1-targeting RNA interference therapy (Figure 8) demonstrated the importance of HMGA1 for HBV expression and replication in this model, corroborating observations made in replicon-transfected and virion-infected cells (Figures 4 and 5). However, clearance of persisting rcccDNA that followed marked repression of viral activities induced by knockdown of HMGA1 (Figure 8 and Supplementary Figure S19) came as a surprise. Viral clearance subsequent to intervention-induced repression has not been commonly observed in this or other models of HBV, or in CHB patients undergoing antiviral therapy. Whether this represents a peculiarity of this model, or a peculiarity of mouse as a surrogate host for HBV, needs to be studied. In addition, HMGA1 knockdown in cell types other than hepatocytes might also play some role in these mice. In view of the dual role played by HMGA1 in HBV life cycle and HCC development, these data suggest HMGA1 as an interesting and promising therapeutic target for treating CHB and CHB-related HCC.

DATA AVAILABILITY

Data that support the findings of this study are available from the corresponding authors upon request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Z.S., Y.X., J.Z. and J.L. designed the experiments and wrote the article. Z.S., J.W., Z.G, S.Z., J.C. and J.H. performed the experiments. Y.G. and J.Z. provided the liver samples of patients. Q.D. contributed to data analyses.

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