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Intrahepatic homeobox protein MSX-1 is a novel host restriction factor of hepatitis B virus

Zhongliang Shen,[1,2] Shenyan Zhang,[1] Zixiang Gao,[2] Xueping Yu,[1,3] Jinyu Wang,[1] Shaokun Pan,[2] Ning Kang,[2] Nannan Liu,[2] Huijun Xu,[2] Mu Liu,[2] Yang Yang,[2] Qiang Deng,[2] Jing Liu,[2,4] Youhua Xie,[1,2,5] Jiming Zhang[1,2]

AUTHOR AFFILIATIONS See affiliation list on p. 17.

ABSTRACT Chronic hepatitis B virus (HBV) infection (CHB) is a risk factor for the development of liver fibrosis, cirrhosis, and hepatocellular carcinoma. Covalently closed circular DNA serves as the sole transcription template for all viral RNAs and viral transcription is driven and enhanced by viral promoter and enhancer elements, respectively. Interactions between transcription factors and these *cis*-elements regulate their activities and change the production levels of viral RNAs. Here, we report the identification of homeobox protein MSX-1 (MSX1) as a novel host restriction factor of HBV in liver. In both HBV-transfected and HBV-infected cells, MSX1 suppresses viral gene expression and genome replication. Mechanistically, MSX1 downregulates enhancer II/core promoter (EnII/Cp) activity via direct binding to an MSX1 responsive element within EnII/Cp, and such binding competes with hepatocyte nuclear factor 4α binding to EnII/Cp due to partial overlap between their respective binding sites. Furthermore, CHB patients in immune active phase express higher levels of intrahepatic MSX1 but relatively lower levels of serum and intrahepatic HBV markers compared to those in immune tolerant phase. Finally, MSX1 was demonstrated to induce viral clearance in two mouse models of HBV persistence, suggesting possible therapeutic potential for CHB.

IMPORTANCE Covalently closed circular DNA plays a key role for the persistence of hepatitis B virus (HBV) since it serves as the template for viral transcription. Identification of transcription factors that regulate HBV transcription not only provides insights into molecular mechanisms of viral life cycle regulation but may also provide potential antiviral targets. In this work, we identified host MSX1 as a novel restriction factor of HBV transcription. Meanwhile, we observed higher intrahepatic MSX1 expression in chronic hepatitis B virus (CHB) patients in immune active phase compared to those in immune tolerant phase, suggesting possible involvement of MSX1 in the regulation of HBV activity by the host. Lastly, intrahepatic overexpression of MSX1 delivered by recombinant adenoviruses into two mouse models of HBV persistence demonstrated MSX1-mediated repression of HBV *in vivo*, and MSX1-induced clearance of intrahepatic HBV DNA in treated mice suggested its potential as a therapeutic target for the treatment of CHB.

KEYWORDS HBV, cccDNA, homeoprotein, HNF4α, transcriptional regulation

Chronic hepatitis B virus (HBV) infection (CHB) is an established risk factor for the development of liver fibrosis, cirrhosis, and hepatocellular carcinoma, causing ~0.9 million HBV-related deaths each year (1).

HBV is an enveloped virus containing a partially double-stranded relaxed circular DNA (rcDNA) (2). In infected hepatocyte, rcDNA is trafficked to the nucleus and converted into covalently closed circular DNA (cccDNA) (2, 3). cccDNA serves as the sole template for the transcription of all viral RNAs, which are driven by four viral promoters, namely core

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Address correspondence to Zhongliang Shen, zhongliangshen11@fudan.edu.cn, Jing Liu, liujing212@fudan.edu.cn, Youhua Xie, yhxie@fudan.edu.cn, or Jiming Zhang, jmzhang@fudan.edu.cn.

Zhongliang Shen, Shenyan Zhang, Zixiang Gao, Xueping Yu, and Jinyu Wang contributed equally to this article. Author order was determined on the basis of seniority.

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promoter (Cp), surface protein promoters 1 and 2 (Sp1 and Sp2), and X promoter (Xp) to produce 3.5, 2.4, 2.1, and 0.7 kb RNAs, respectively (2, 4). Viral transcription is also modulated by two enhancers (EnI and EnII) in a position- and orientation-independent manner. EnII is located upstream of basal core promoter and overlaps with Cp, whereas EnI is located upstream of and overlaps with Xp. Cp-driven transcription produces both the 3.5-kb pregenomic (pg) RNA and the 3.5-kb preC RNA. The pgRNA functions as translation template for polymerase (Pol) and core protein (HBc), and also can be bound by Pol to initiate viral replication that continues inside capsid formed by HBc. The preC RNA functions as translation template of secreted e antigen (HBeAg) (2). Transcription of the viral 2.4/2.1 and 0.7 kb RNAs is driven by Sp1/Sp2 and Xp promoters, and the products direct translation of large/middle/small surface antigens (L/M/SHBsAg) and X protein (HBx), respectively (2).

Natural history of CHB is traditionally divided into four phases on the basis of different viral and host immune status (5): immune tolerant (IT), immune active (IA), inactive carrier (IC), and HBeAg-negative hepatitis (ENH) phases. Typical IT phase is characterized by the positive serum HBeAg, very high levels of serum HBV DNA (>10^7 IU/mL), and minimal or no liver necroinflammation as reflected by normal-to-low serum alanine aminotransferase (ALT) levels. IA phase is characterized by positive serum HBeAg, relatively high levels of serum HBV DNA (10^4–10^7 IU/mL), and moderate-to-severe inflammation in liver (hepatitis) with elevated ALT levels. Patients in IT phase may enter IA phase autonomously, and those infected during adulthood tend to do so more rapidly. Intrahepatic immune responses in IA phase have been shown to play important roles in controlling HBV (5, 6), for instance, through removal of infected hepatocytes by HBV-specific cytotoxic T lymphocytes. In contrast, non-immune host factors and mechanisms that might contribute toward such control are less well understood.

Homeobox protein MSX-1 (MSX1) is a transcriptional repressor in the muscle segment homeobox gene family and plays key roles in embryogenesis and development by inhibiting gene expression through interactions with components of the core transcription complex and other homeoproteins (7–9). Although MSX1's homeobox (HO) domain has been shown to mediate its binding to specific DNA sequences in promoters of target genes (10, 11), MSX1-mediated transcriptional repression independent of HO domain has also been observed (12, 13). In host-pathogen interactions, MSX1 has been shown to be essential for modulating innate immune response against certain RNA viruses including Sendai virus and vesicular stomatitis virus (14). Our previous data showed higher liver MSX1 mRNA levels in CHB patients in IA phase compared to those in IT phase (15), and a more prominent increase in MSX1 mRNA was also associated with better response to IFNα treatment in CHB patients (16), suggesting possible involvement of MSX1 in host-virus interactions.

Here, we report data demonstrating that MSX1 functions as a novel host restriction factor that represses HBV transcription in liver cells. Mechanistically, MSX1 inhibits EnII/Cp activity by competing with the transcriptional upregulator hepatocyte nuclear factor 4α (HNF4α) for binding to overlapped binding sites within EnII/Cp. Analysis of liver biopsy specimen also revealed higher MSX1 expression levels in IA phase compared to IT phase. Finally, MSX1 overexpression delivered by recombinant viral vectors induced viral clearance in two mouse models of HBV.

RESULTS

MSX1 suppresses HBV gene expression and genome replication in replicon-transfected and virus-infected cells

To investigate the effects of MSX1 on viral gene expression and genome replication, a previously established recombinant cccDNA (rcccDNA) system was utilized (17). In this system, rcccDNA is produced from transfected precursor rcccDNA (prcccDNA) plasmid through Cre-mediated recombination and is highly similar to wild-type cccDNA except for a small extraneous intron that is removed post-transcriptionally through RNA splicing. In Huh7 cells transfected with prcccDNA and Cre-expressing plasmids, co-transfection

with Flag-tagged MSX1-expressing plasmid (pMSX1-Flag) markedly decreased intracellular HBV transcripts (3.5 and 2.4/2.1 kb RNAs), replication intermediates (capsid-associated DNA) and intracellular viral proteins (HBx and HBc), as well as secreted viral antigens (HBsAg and HBeAg) (Fig. 1A). In contrast, exogenous MSX1 did not affect Cre-mediated rcccDNA production (Fig. 1A). Conversely, knockdown of endogenous MSX1 expression via co-transfection with plasmid expressing short hairpin RNA targeting MSX1 (pshMSX1) elevated both HBV gene expression and genome replication without affecting rcccDNA level (Fig. 1B). To further confirm MSX1-mediated HBV repression, endogenous MSX1 in Huh7 cells was stably knocked down through transduction of shMSX1-expressing lentivirus (Lenti-shMSX1). As expected, knockdown of MSX1 increased HBV gene expression and replication, while rescue of MSX1 expression via co-transfection with pMSX1-Flag reinstated inhibition of HBV (Fig. 1C). Meanwhile, cell viability assay revealed

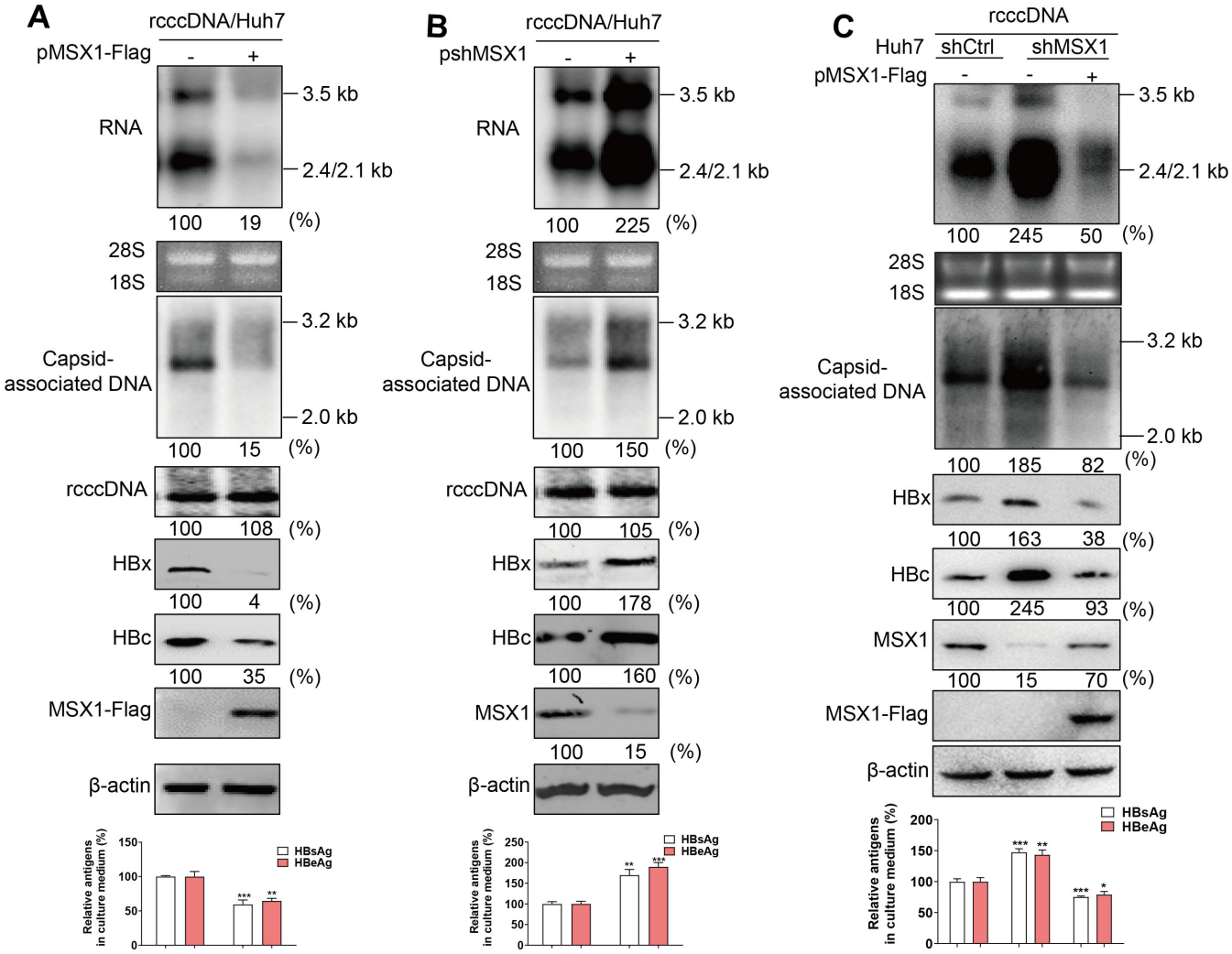

FIG 1 MSX1 suppresses HBV gene expression and genome replication in Huh7 cells. Huh7 cells cultured in 6-well plates were transfected with 1 µg of prcccDNA and 1 µg of pCMV-Cre, plus 1 µg of pMSX1-Flag or vector control plasmid (pCtrl) (A) or pshMSX1 or pshCtrl (B). At day 3 post transfection, intracellular HBV RNA, capsid-associated DNA, and rcccDNA were assayed by Northern and Southern blots, respectively. Exogenously and endogenously expressed MSX1 were determined in Western blot using Flag and MSX1 antibodies, respectively. Intracellular viral proteins (HBc and HBx) and secreted antigens (HBsAg and HBeAg) were examined using Western blot and ELISA, respectively. (C) Huh7 cells were transduced with shMSX1-expressing lentivirus or control virus (Lenti-shMSX1 or Lenti-shCtrl) to obtain cells with stable knockdown of MSX1 expression [Huh7(shMSX1)] and control cells [Huh7(shCtrl)], respectively. These cells were co-transfected with prcccDNA, pCMV-Cre, and pMSX1-Flag or pCtrl. HBV gene expression and genome replication were determined as in A and B. HBV RNA, DNA, and protein levels were quantified using densitometry scanning and normalized using respective control as 100%. Group means and SEMs of normalized antigen levels were presented, and significances calculated using unpaired two-tailed *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

that MSX1-mediated suppression of HBV was not due to cytotoxic effects (data not shown).

Next, inhibitory effects of MSX1 on HBV were evaluated in infection models. HBV-infected HepG2-NTCP cells (hepatoma HepG2 cells stably transfected with HBV receptor NTCP) and primary human hepatocytes (PHH) were transduced with MSX1-Flag- or shMSX1-expressing lentivirus. A negative correlation between MSX1 protein levels and HBV transcription, viral replication, and antigen production was observed in both systems (Fig. 2), similar to data obtained in transfected cells (Fig. 1). Taken together, these data demonstrated MSX1 as a novel host restriction factor of HBV.

MSX1 downregulates EnII/Cp activity via direct binding to an MSX1 responsive element within EnII/Cp

Marked reduction of HBV transcripts by MSX1 overexpression (Fig. 1A and 2A) suggested possible involvement of this transcriptional repressor in the regulation of HBV transcription. Indeed, reporter assay in Huh7 cells showed that transcription driven by EnII/Cp, but not Sp1, Sp2, or EnI/Xp, was repressed by MSX1 (Fig. 3A and B). Furthermore, enhancer activity of EnII/Cp when placed downstream of reporter-coding region to enhance transcription from Sp1, Sp2, and EnI/Xp was also inhibited by MSX1 (Fig. 3C). Similar results were obtained in HepG2 cells (data not shown).

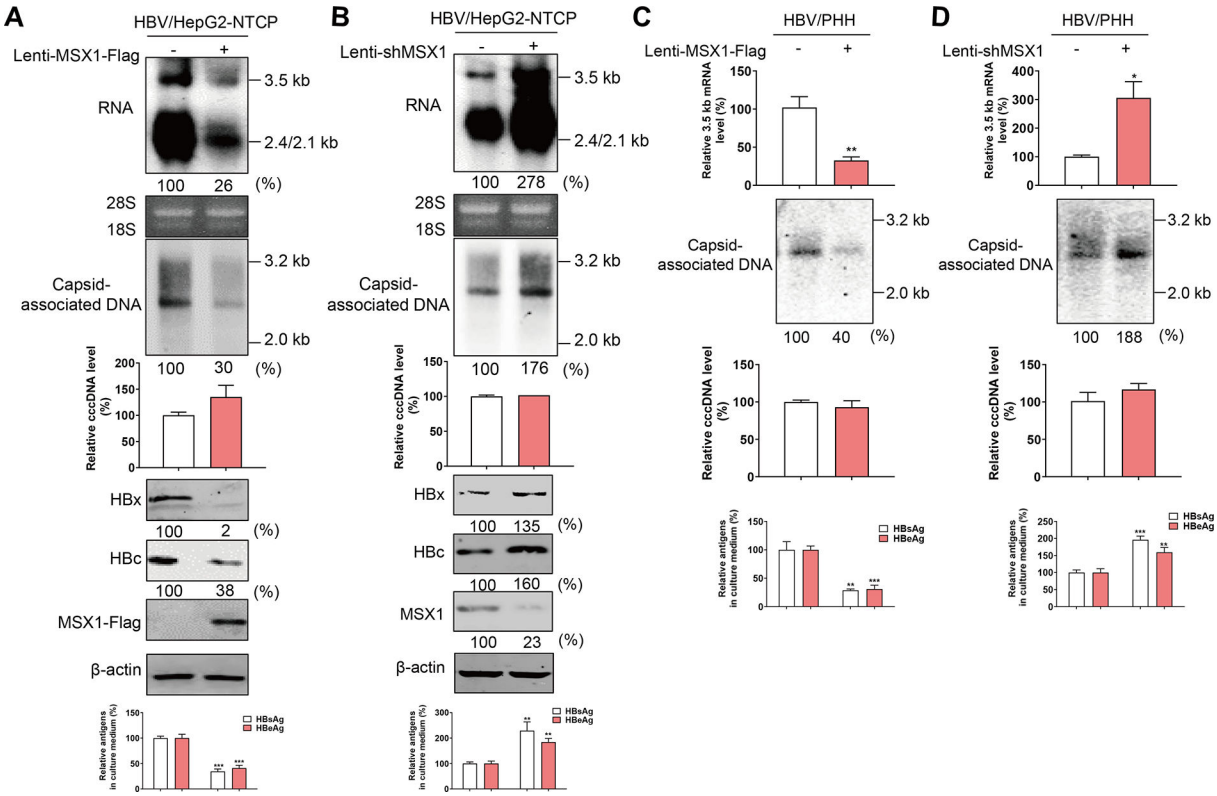

FIG 2 MSX1 suppresses HBV gene expression and genome replication in HBV-infected cells. HepG2-NTCP (A and B) and PHH (C and D) cells were infected with HBV virions at a multiplicity of infection of 1,000 geq/cell overnight, and culture media were changed the next day. At day 2, HepG2-NTCP cells were transduced by MSX1-Flag-expressing lentivirus or control virus (Lenti-MSX1-Flag or Lenti-Ctrl) (A), or shMSX1-expressing lentivirus or control virus (Lenti-shMSX1 or Lenti-shCtrl) (B). At day 7, intracellular HBV RNA, capsid-associated DNA, and cccDNA were examined using Northern and Southern blots, and qrtPCR, respectively. Exogenously and endogenously expressed MSX1 were determined in Western blot using Flag and MSX1 antibodies, respectively. Intracellular viral proteins (HBc and HBx) and secreted antigens (HBsAg and HBeAg) were examined using Western blotting and ELISA, respectively. At day 5, PHH cells were transduced by Lenti-MSX1-Flag or Lenti-Ctrl (C), or Lenti-shMSX1 or Lenti-shCtrl (D). At day 9, intracellular HBV RNA was analyzed using qrtPCR. Capsid-associated DNA, cccDNA, and secreted antigens were determined as in A and B. HBV RNA, DNA, and protein levels were quantified using densitometry scanning and normalized using respective control as 100%. Group means and SEMs of normalized values were presented, and significances calculated using unpaired two-tailed *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. geq, genome equivalents.

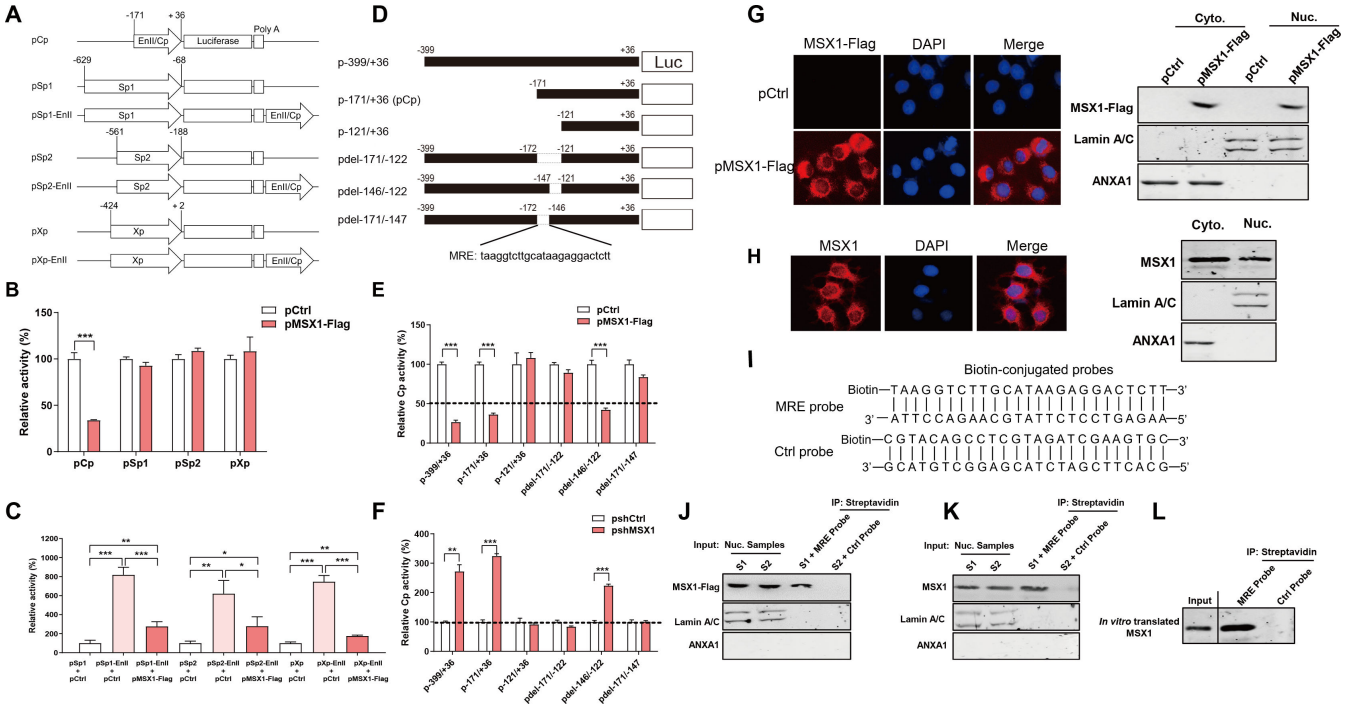

FIG 3 MSX1 downregulates EnII/Cp activity via binding to an MSX1 responsive element (MRE) within EnII/Cp. (A) Schematic representation of HBV enhancer/promoter firefly luciferase reporter plasmids. Numbers denote nucleotide positions relative to the first nucleotide of corresponding open reading frame. EII/Cp was cloned into Sp1, Sp2, and Xp reporter plasmids downstream of polyA signal in the same direction as upstream promoter to test its enhancer activity. (B) Huh7 cells cultured in 24-well plates were transfected with 0.4 µg of Cp, Sp1, Sp2, or Xp reporter plasmid, 0.4 µg of pMSX1-Flag or pCtrl, and 0.1 µg of pRL-TK. At 48 h post transfection, cells were lysed and subjected to dual-luciferase reporter assay. (C) The activities of Sp1, Sp2, and Xp in the presence of downstream EnII/Cp when co-transfected with or without pMSX1-Flag were similarly determined in Huh7 cells. (D) Schematic representation of EnII/Cp firefly luciferase reporter plasmid (p−399/+36) and its serial deletion mutants. p−399/+36 contains both EnII/Cp (Cp, nt −171 to +36) and the upstream negative regulatory element (nt −172 to −399) sequences. Numbers denote nucleotide positions relative to the first nucleotide of PreC open reading frame. Deleted nucleotide sequences are represented as dashed lines. Huh7 cells cultured in 24-well plates were co-transfected with 0.4 µg of p−399/+36 or its deletion mutant, 0.4 µg of pMSX-Flag or pCtrl (E), or 0.4 µg of pshMSX1 or pshCtrl (F), and 0.1 µg of pRL-TK. At 48 h post transfection, relative promoter activities were measured. Group means and SEMs of normalized firefly versus Renilla luciferase activity ratios were presented, and significances calculated using unpaired two-tailed *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Intracellular distribution of exogenous MSX1 (G, left panel) and endogenous MSX1 (H, right panel) was analyzed in immunofluorescence using Flag and MSX1 antibodies, respectively, and nucleocytoplasmic separation coupled with Western blot (G and H, right panels). Lamin A/C and annexin A1 (ANXA1) were used as markers of nuclear and cytoplasmic fractions, respectively. (I) Biotinylated MRE (nt −171 to −147 of Cp) and scrambled sequence control probes (Ctrl probe) used in DNA pulldown assay. Chemically synthesized probes were incubated with nuclear protein extracts from pMSX1-Flag-transfected (J) or untreated Huh7 cells (S1 and S2) (K), or *in vitro* translated MSX1 (L), and streptavidin-coupled beads. After washing, beads were heated and subjected to Western blot.

In order to map potential MSX1 responsive element(s) (MRE), EnII/Cp (nucleotides/nt −171 to +36 in relation to preC start codon) as well as upstream negative regulatory elements (NRE, nt −399 to −172) (18) were used to generate reporter plasmid p−399/+36. A series of deletions were then introduced into p−399/+36 (Fig. 3D), and reporter assay mapped MSX1 responsiveness to a segment spanning nt −171 to nt −147 (Fig. 3E and F). Since immunofluorescence and nuclear/cytoplasmic fractionation assays showed that endogenous as well as exogenous MSX1 was localized in both cytoplasm and nucleus (Fig. 3G and H), potential physical association between MSX1 and MRE was analyzed using biotinylated double-stranded DNA probe pulldown assay (Fig. 3I). As shown in Fig. 3J and K, MRE probe, but not the scrambled control probe (Ctrl probe), effectively pulled down both exogenous and endogenous MSX1 in nuclear extracts, as well as *in vitro* translated MSX1 (Fig. 3L).

We went on to test whether MSX1 also binds to MRE in the context of HBV genome. In prcccDNA-transfected cells, chromatin immunoprecipitation (ChIP) followed by Southern

blot showed that both exogenous and endogenous MSX1 were recruited onto rcccDNA (Fig. 4A), while deletion of MRE from rcccDNA precursor reduced MSX1 occupancy by nearly 80% (Fig. 4B). On the other hand, while HBV infection did not change the expression level or subcellular localization of endogenous MSX1 in HepG2-NTCP cells (Fig. 4C and D), MSX1 occupancy on nuclear cccDNA was confirmed by ChIP-qPCR in both HBV-infected HepG2-NTCP and PHH cells (Fig. 4E and F). Taken together, these results showed that MSX1 directly binds MRE within EnII/Cp and represses both its promoter and enhancer activities.

MSX1 represses EnII/Cp by competing with HNF4α for MRE binding

Within EnII/Cp, MRE (nt −171 to −147) encompasses the entire Box α (nt −168 to −146) (18) and overlaps with C/EBP and 5′ half of HNF4α-binding sites (Fig. 5A). Since HNF4α

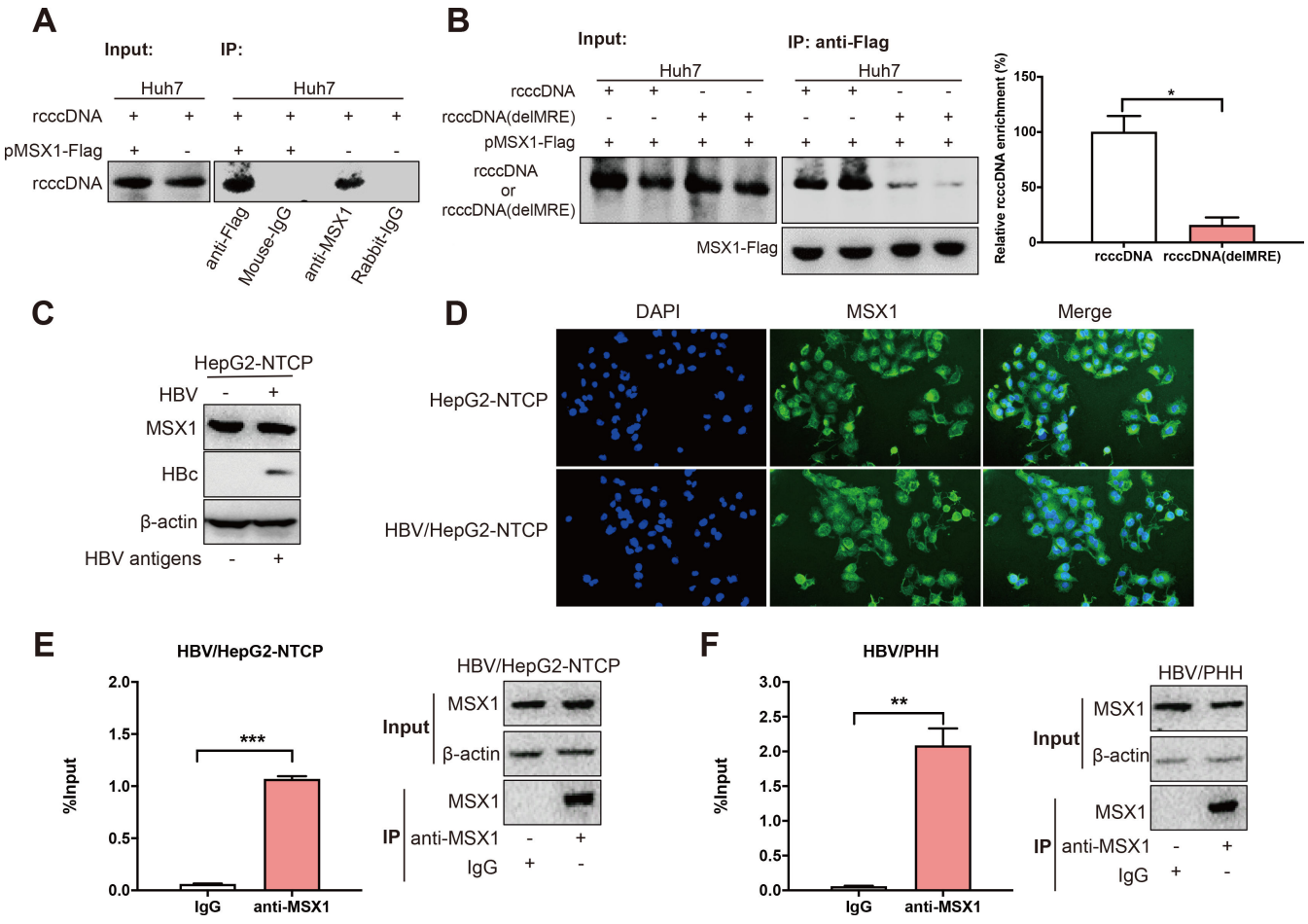

FIG 4 MSX1 is recruited to recombinant and authentic cccDNA. (A) Huh7 cells cultured in 6-well plates were transfected with 1 µg of prcccDNA, 1 µg of pCMV-Cre, and 1 µg of pMSX1-Flag or pCtrl. At day 3 post transfection, ChIP assay was performed, and rcccDNA immunoprecipitated by Flag antibody, MSX1 antibody, mouse or rabbit control IgG was subjected to Southern blot. (B) ChIP assay followed by Southern blot was similarly performed on Huh7 cells transfected with prcccDNA or MRE-deleted prcccDNA [prcccDNA(delMRE)], pCMV-Cre, and pMSX1-Flag. Exogenous MSX1 immunoprecipitated by Flag antibody was determined in Western blot using MSX1 antibody. Immunoprecipitated rcccDNA and rcccDNA(delMRE) signals were scanned using Image J software and normalized against rcccDNA signal density. (C and D) HepG2-NTCP cells were infected with HBV virions at a multiplicity of infection of 1,000 geq/cell overnight. Cells were washed with phosphate buffer saline the next day, and then the culture media changed. At day 7 post infection, the expression levels of endogenous MSX1 and HBc were analyzed in Western blot using MSX1 and HBc antibodies, respectively, with HBV antigens (HBsAg and HBeAg) in culture media indicated. Intracellular distribution of endogenous MSX1 was analyzed in immunofluorescence using MSX1 antibody. HBV-infected HepG2-NTCP (E) and PHH cells (F) were subjected to ChIP assay, and cccDNA immunoprecipitated by MSX1 antibody or rabbit control IgG was quantitated in qrtPCR and indicated as percentage of input (E and F, left panels), and immunoprecipitated MSX1 was determined using Western blot (E and F, right panels). Group means and SEMs within group were presented, and significances calculated using unpaired two-tailed *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. geq, genome equivalents.

is a nuclear factor essential for Cp activity (19), we examined whether HNF4α played any role in MSX1-mediated repression of EnII/Cp via MRE binding. Endogenous HNF4α expression in Huh7 cells, or its absence in human embryonic kidney 293T (HEK293T) cells, was not affected by MSX1 overexpression (Fig. 5B). In both cell lines, HNF4α co-transfection increased reporter expression from p−399/+36, whereas MSX1-mediated repression was only observed in Huh7 but not in HEK293T cells. On the other hand, MSX1 co-transfection with HNF4α markedly reduced transcriptional upregulation mediated by the latter in both cell lines (Fig. 5C through E). More importantly, deletion of MRE obliterated regulation by HNF4α, MSX1, or both (Fig. 5F and G). Conversely, MSX1 mutants with deletion in the DNA-binding homeobox (HO) domain, and in particular the a.a. 218–260 segment, displayed reduced or obliterated repression of EnII/Cp promoter activity (Fig. 5H through K). These data suggested that MSX1-mediated repression of EnII/Cp depends on its binding to MRE but also involves HNF4α.

As no interaction between MSX1 and HNF4α was observed in co-immunoprecipitation (Co-IP) assay (Fig. 5L), we tested their binding to MRE. As shown in Fig. 5M, exogenous MSX1 and endogenous HNF4α bound MRE in a mutually exclusive fashion

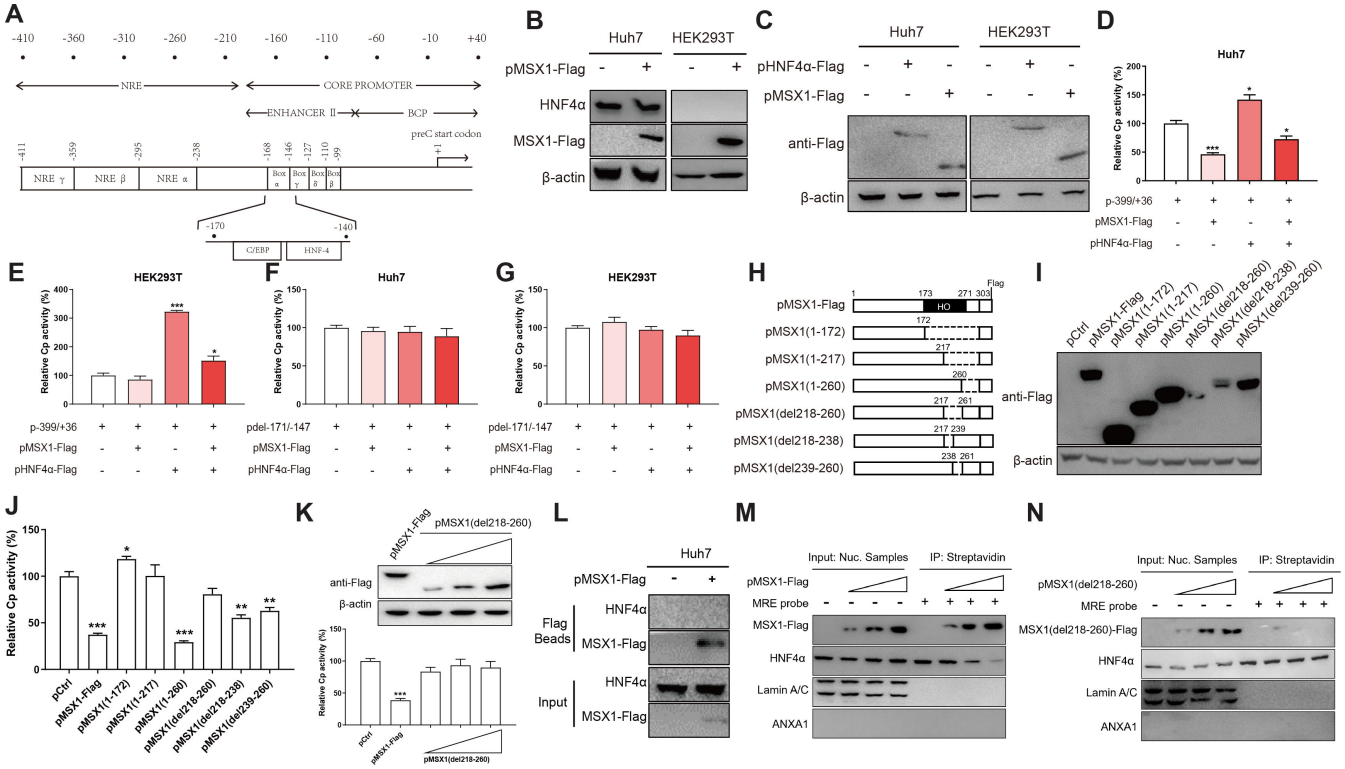

FIG 5 MSX1 competes with HNF4α for binding to MRE. (A) Schematic representation of EnII/Cp and the upstream NRE. C/EBP- and HNF4α-binding sites within Box α and γ are depicted. Numbers denote nucleotide positions relative to the first nucleotide of PreC open reading frame. (B and C) Huh7 and HEK293T cells were transfected with pMSX1-Flag or Flag-tagged HNF4α-expressing plasmid (pHNF4α-Flag) or pCtrl. At day 2 post transfection, exogenous MSX1 and HNF4α and endogenous HNF4α expression were determined in Western blot using Flag and HNF4α antibodies, respectively. Huh7 (D and F) and HEK293T (E and G) cells cultured in 24-well plates were co-transfected with 0.4 µg of Cp reporter plasmid p−399/+36 (nt −399 to +36 relative to PreC) or pdel−171/−47 (nt −171 to −47 deleted in p−399/+36), 0.4 µg of pMSX1-Flag or pCtrl, 0.4 µg of pHNF4α-Flag or pCtrl, and 0.1 µg of pRL-TK. At 48 h post transfection, promoter activities were measured using dual-luciferase reporter assay. (H) Schematic representation of pMSX1-Flag and its serial deletion mutants. Deleted region within MSX1 is represented by dashed lines. Numbers denote amino acid positions. Western blot and dual-luciferase reporter assays were performed to analyze the expression of wild-type and mutant MSX1 (I) and their effects on Cp (p−399/+36) activities (J) in Huh7 cells, respectively, and the dosage effects of MSX1(del218-260) on Cp activities in Huh7 cells as well (K). Group means and SEMs of normalized firefly versus Renilla luciferase activity ratios were presented, and significances calculated using unpaired two-tailed *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (L) Huh7 cells were transfected with pMSX1-Flag or pCtrl. Interaction between MSX1-Flag and HNF4α was analyzed using co-immunoprecipitation assay. Nuclear proteins were extracted from Huh7 cells transfected with an increasing amount of pMSX1-Flag (M) or pMSX1(del218-260) (N) and then incubated with biotinylated MRE probe and streptavidin-coupled beads. After washing, beads were heated and subjected to Western blot. Lamin A/C and annexin A1 (ANXA1) were used as markers of nuclear and cytoplasmic fractions, respectively.

in biotinylated DNA pulldown assay, suggesting competition between the two for MRE binding. Furthermore, the MSX1 mutant with the a.a. 218–280 segment essential for repression deleted [MSX1(del218-260)] failed to bind MRE or reduce HNF4α's binding to MRE. Collectively, these data demonstrated that MSX1 competes with the positive regulator HNF4α for binding to MRE, and reduced HNF4α recruitment to EnII/Cp would account for the low promoter/enhancer activity.

Fine mapping of MSX1-binding site within MRE

Being a key positive regulator of EnII/Cp, HNF4α targeting by other (co-)regulators is not surprising. For instance, a recent study reported that, similar to MSX1-mediated repression described above, host transcription factor MafF suppresses EnII/Cp activity by competing with HNF4α for binding to overlapping sequences in EnII/Cp (20). Close examination of MRE in relation to HNF4α- and MafF-binding sites showed that MRE overlaps with the 5′ half of HNF4α-binding site, whereas the reported MafF-binding site overlaps with the 3′ half of HNF4α-binding site and overlaps with MRE by only one nucleotide (Fig. 6A). In addition, no interaction between MSX1 and MafF was observed in co-IP assay (Fig. 6B), and MafF could not be pulled down by biotin-labeled MRE probe (Fig. 6C). Therefore, MSX1 and MafF most likely compete with HNF4α binding independently from each other.

As further evidence of direct competition between MSX1 and HNF4α for binding to MRE, serial 5-nt mutations were introduced to MRE probes (Fig. 6A), and DNA pulldown assay mapped MSX1 binding to the 3′-terminal 15 bp (nt −161 to −147) that overlaps

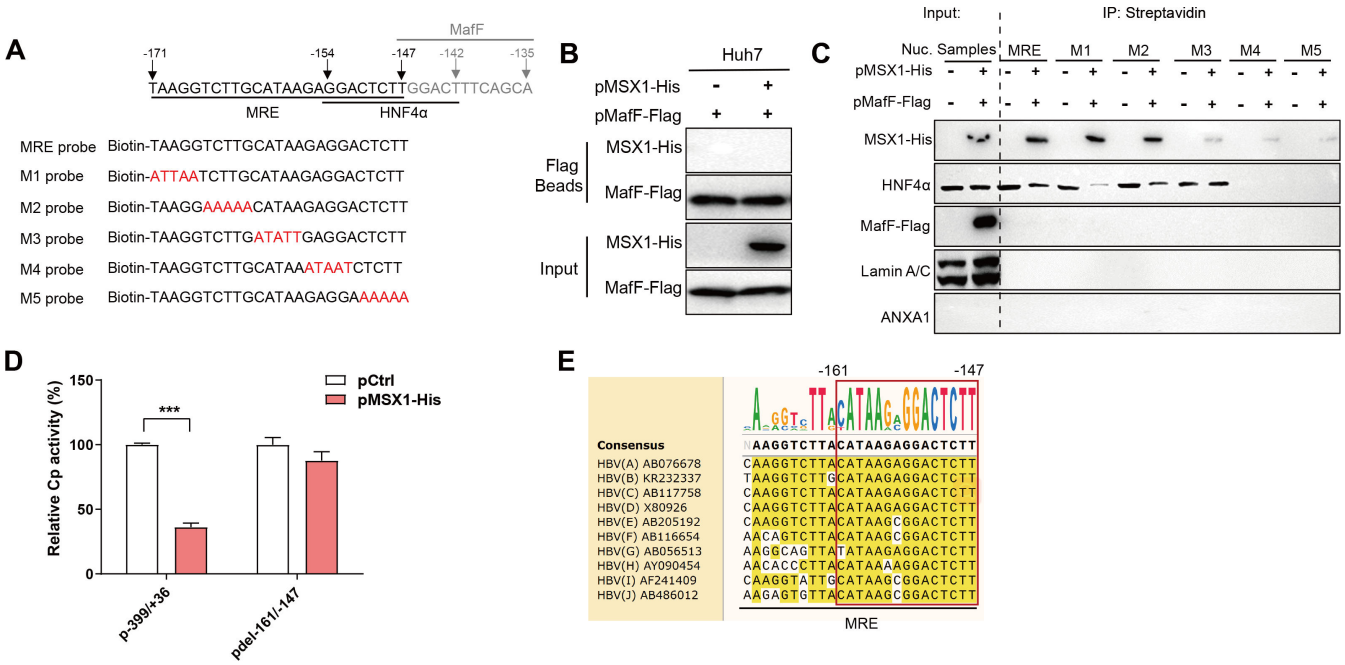

FIG 6 Fine mapping of MSX1's binding site within MRE. (A) Schematic presentation of MRE and the binding sites for HNF4α and MafF. Numbers denote nucleotide positions relative to the first nucleotide of PreC open reading frame. Wild-type biotinylated MRE and its mutant probes were chemically synthesized and used in DNA pulldown assay. Mutant nucleotide sequences were highlighted in red. (B) Huh7 cells were co-transfected with Flag-tagged MafF-expressing plasmid (pMafF-Flag) and His-tagged MSX1-expressing plasmid (pMSX1-His) or pCtrl. Interaction between MafF-Flag and MSX1-His was analyzed using Co-IP assay. (C) Huh7 cells cultured in 10 cm dish were co-transfected with 7.5 µg of pMSX1-His and 7.5 µg of pMafF-Flag, or 15 ug of pCtrl. At day 2 post transfection, nuclear proteins extracted from Huh7 cells were divided evenly into six samples and then incubated with indicated DNA probes and streptavidin-coupled beads. After washing, beads were heated and subjected to Western blot. (D) Huh7 cells cultured in 24-well plates were co-transfected with 0.4 µg of Cp reporter plasmid p−399/+36 (nt −399 to +36 relative to PreC) or pdel−161/−147 (nt −161 to −147 deleted in p−399/+36), 0.4 µg of pMSX1-His or pCtrl, and 0.1 µg of pRL-TK. At 48 h post transfection, promoter activities were measured using dual-luciferase reporter assay. (E) Alignment of MRE sequences of representative strains of currently recognized HBV genotypes (A–J). MSX1's binding site sequences (nt −161 to −147) are highlighted in red box.

with the 5′ half of HNF4α-binding site (nt −154 to −147) (Fig. 6A and C). Reporter assay confirmed that deletion of nt −161 to −147 rendered EnII/Cp irresponsive to MSX1 repression (Fig. 6D). MRE sequences, especially the nt −161 to −147 segment, are highly conserved in known HBV genotypes (A–J), suggesting that MSX1-mediated repression is not genotype specific (Fig. 6E).

Analysis of MSX1 expression profile in IT and IA phase CHB patients

In our previous study using liver biopsy specimens from patients in IT and IA phases, RNA microarray analysis revealed MSX1 as one of the differentially expressed genes with elevated expression in IA samples (Fig. 7A) (15). To assess MSX1 expression at protein level, biopsy samples from 20 IT and IA patients (Table 1) were subjected to immunohistochemistry and Western blot analysis. Patients in IA phase had lower serum HBV DNA levels and elevated ALT levels compared to those in IT phase (Table 1). Immunohistochemistry results showed that HBcAg was predominantly localized in the nuclei in both IA and IT phase biopsy samples, but IT samples displayed comparatively higher HBcAg expression levels (Fig. 7B through D). In contrast, MSX1 staining was relatively higher in IA samples compared to IT samples despite similar subcellular distribution. These patterns of HBcAg and MSX1 expression were confirmed using Western blot on the same samples (Fig. 7E through G). Taken together with MSX1-mediated repression of HBV demonstrated *in vitro* (see above), these data suggested the possibility that elevated MSX1 expression and consequently its repression of HBV might constitute part of host response to chronic infection in IA phase.

Human MSX1 delivered by adeno-associated virus induced viral repression and clearance in mouse models of HBV persistence

Mouse and human HNF4α have nearly identical amino acid sequences, and murine HNF4α apparently plays similarly essential role in positive regulation of HBV in mouse hepatocytes (21). Mouse models of HBV replication could therefore serve as a testing platform for MSX1's effects on HBV *in vivo*. Reporter assay indeed showed that human MSX1 markedly repressed EnII/Cp activity in two murine hepatoma cell lines Hepa1-6 and AML12 (Fig. 8A and B). Similar results were obtained from *in vivo* reporter assay, wherein C57BL/6 mice pre-injected with MSX1-expressing recombinant adenovirus (AAV-MSX1-Flag) or control virus (AAV-Ctrl) were hydrodynamically injected (HDI) with reporter plasmids via tail vein, and intrahepatic reporter expression was analyzed 2 days later (Fig. 8C and D).

Next, effects of AAV-MSX1-Flag administration on HBV were tested using two mouse models of HBV persistence. In the model based on rcccDNA (22), Cre-transgenic C57BL/6 mice were injected with adenovirus-carrying precursor rcccDNA (Adv/prcccDNA) via tail vein, and mice that remained positive for serum HBV antigens (HBsAg and HBeAg) by 4 weeks post injection were taken as displaying rcccDNA persistence and injected with AAV-MSX1-Flag or AAV-Ctrl (Fig. 9A). As shown in Fig. 9B and D, in AAV-MSX1-Flag-injected mice, serum HBsAg and HBeAg levels decreased much more quickly compared to AAV-Ctrl-injected mice and became undetectable in two out of six mice by 10 weeks post injection of AAV (w.p.i.), while both viral markers persisted at relatively high levels in all AAV-Ctrl-injected mice (n = 6). MSX1-induced HBsAg clearance was not accompanied by HBsAg antibody (HBsAb) development (Fig. 9C). At 10 w.p.i., mice were sacrificed, and analyses using liver tissues showed that levels of intracellular HBV capsids, capsid-associated replication intermediates, and rcccDNA were generally lower in AAV-MSX1-Flag-injected mice (Fig. 9E). Notably, the two AAV-MSX1-injected mice that achieved clearance of serum HBV antigens had non-detectable intrahepatic rcccDNA (Fig. 9E), indicating sterile cure instead of mere transcriptional repression. Highly comparable results were obtained (Fig. 10) using another mouse model of HBV persistence based on 1.3× genome replicon derived from a genotype B isolate (termed BPS) (23). In this model, however, three out of six AAV-MSX1-Flag-injected mice cleared serum and intrahepatic markers, and one of the three developed serum HBsAb in this process (Fig. 10).

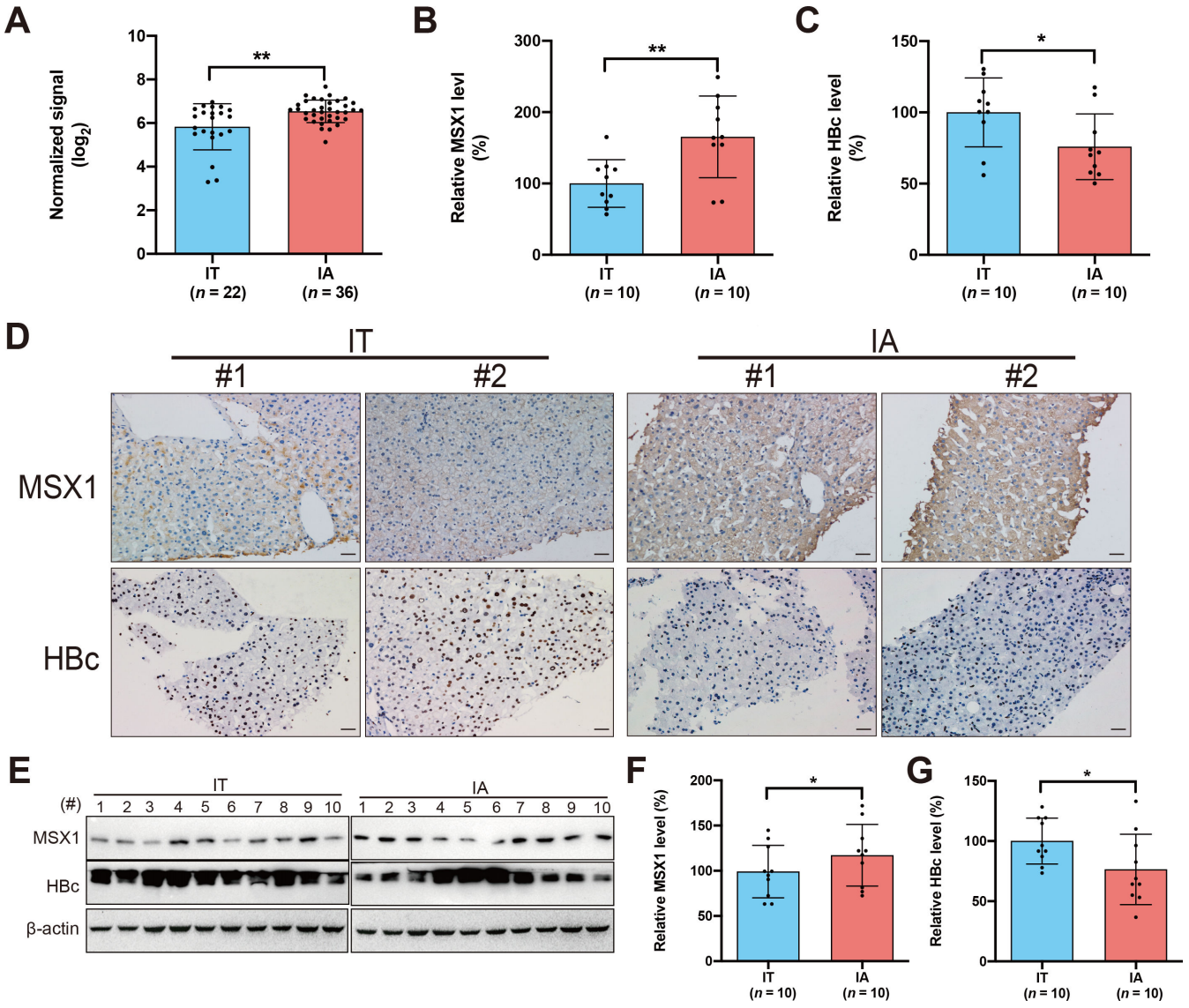

FIG 7 MSX1 expression profiles in immune tolerant and immune active phase CHB patients. (A) Liver biopsy samples were collected from patients in IT and IA phases with group size indicated (*n*) and then subjected to RNA extraction and microarray analysis as described in our previous study (15). Biopsy samples from 20 IT and IA patients (Table 1) were assayed for MSX1 protein expression. Immunochemistry staining of MSX1 (B) and HBc (C) proteins was performed using antibodies against MSX1 and HBc, respectively, and representative images were presented (D). Protein levels were evaluated using H-SCORE analysis. MSX1 and HBc protein levels were additionally evaluated using Western blot (E) and quantified using densitometry scanning (F and G). Group means and SEMs were presented, and significances calculated using unpaired two-tailed *t*-test. *, $P < 0.05$; **, $P < 0.01$. Scale bars: 100 µm.

Collectively, these results indicated that exogenously administered MSX1 not only represses HBV gene expression and genome replication in mice, but such repression enabled at least some of the treated mice to clear HBV-harboring cells in liver to achieve viral clearance.

DISCUSSION

Due to its essential roles in HBV gene expression and replication, modulation of EnII/Cp activity tends to achieve significant effects on viral life cycle. Here, we report that MSX1, one of host homeoprotein transcription factors, inhibits HBV RNA transcription, antigen expression, and genome replication in HBV-transfected and -infected cells (Fig. 1 and 2). It was then revealed that MSX1 downregulates both the promoter and enhancer

TABLE 1 Clinical and virological information of CHB patients

	Immune tolerant (n = 10)	Immune active (n = 10)
Age (years)[a]	28 (22–30)	39 (25–61)
Gender (M/F)	6/4	7/3
HBV DNA (log_{10} copies/mL)	7.53 (7.34–9.18)	6.44 (3.25–8.41)
HBsAg (+/–)	10/0	10/0
HBeAg (+/–)	10/0	10/0
ALT (U/L)[a]	29 (13–33)	173 (72–1,440)
Inflammation		
G0/G1/G2/G3/G4	5/5/0/0/0	0/2/4/3/1
Fibrosis		
S0/S1/S2/S3/S4	4/5/1/0/0	0/1/5/2/2

[a]Expressed as X ± SD. Inflammation and fibrosis were graded using blinded liver biopsy sections by qualified pathologists according to the standards of Scheuer system.

activities of EnII/Cp and acts via direct binding to an MSX1 responsive element within EnII/Cp (Fig. 3 and 4). Previous studies have identified host gene targets that are transcriptionally repressed by MSX1 (7, 9, 13). To our knowledge, this is the first report of MSX1-repressing viral transcription.

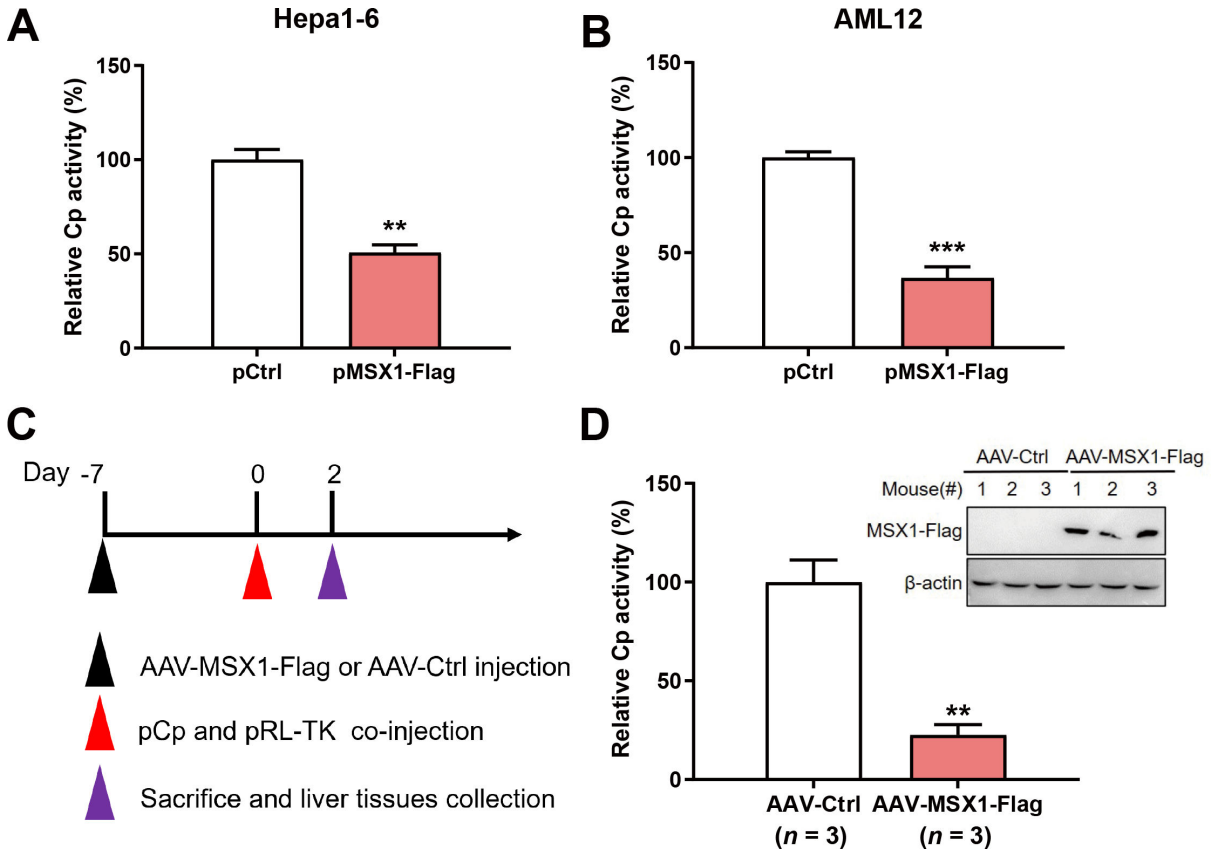

FIG 8 Human MSX1 represses EnII/Cp activity in mouse cells *in vitro* and *in vivo*. Murine hepatoma Hepa1-6 (A) and AML12 (B) cells cultured in 24-well plates were co-transfected with 0.4 µg of pCp, 0.4 µg of pMSX1-Flag or pCtrl, and 0.1 µg of pRL-TK Renilla luciferase plasmid. At 48 h post transfection, Cp activities were measured using dual-luciferase reporter assay. (C) C57BL/6 mice were pre-injected with 2×10^{11} genome equivalents of AAV-MSX1-Flag or control virus (AAV-Ctrl) via tail vein. At day 7 post injection of AAV, mice were injected with 2 µg of pCp and 0.5 µg of pRL-TK via HDI method. At day 2 post injection of plasmids, mice were sacrificed to collect liver tissues. (D) Intrahepatic Cp activities and exogenously expressed human MSX1 were measured using dual-luciferase reporter assay and Western blot, respectively. Group means and SEMs of normalized firefly versus Renilla luciferase activity ratios were presented with group sizes (n) indicated, and significances calculated using unpaired two-tailed t-test. **, $P < 0.01$; ***, $P < 0.001$.

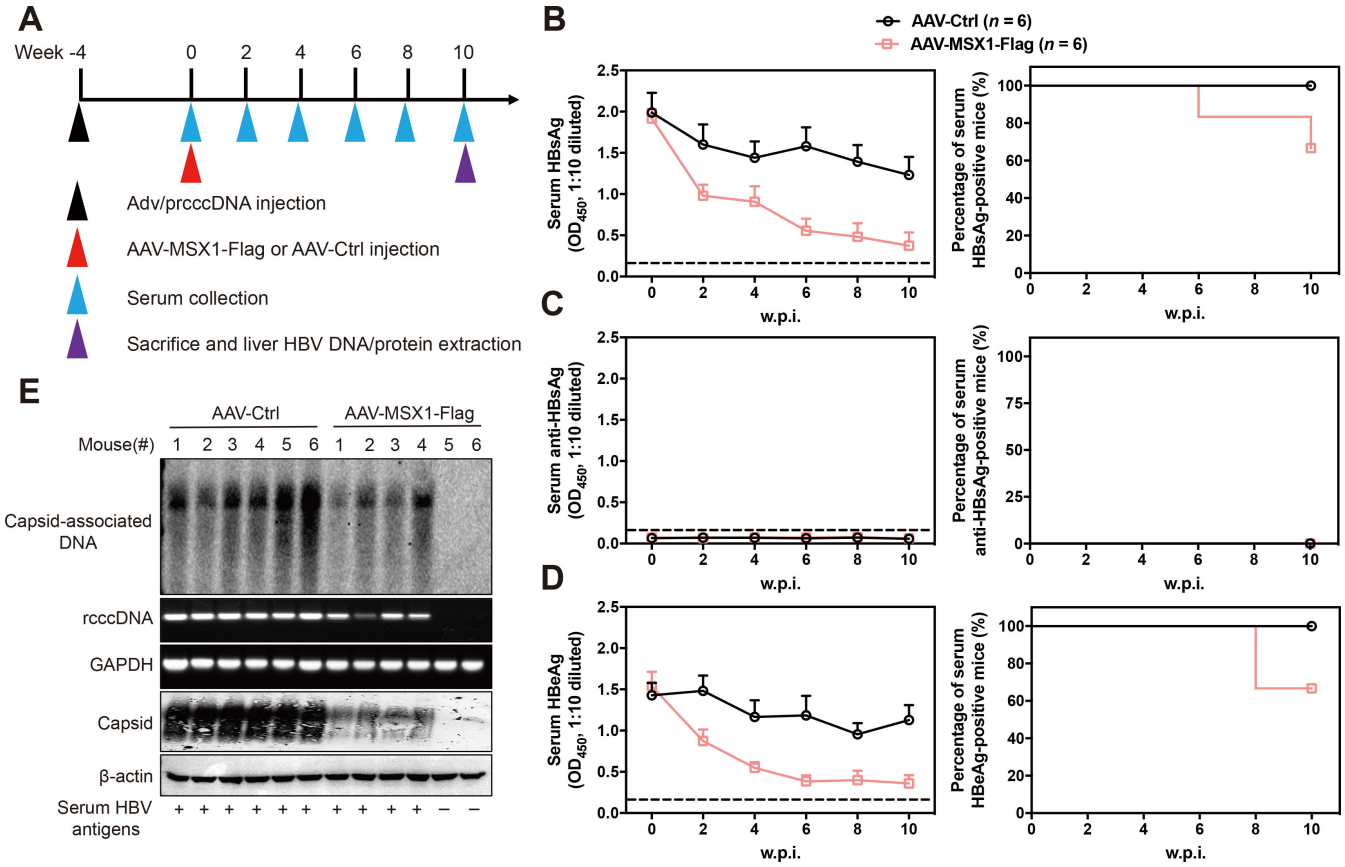

FIG 9 Effects of human MSX1 administration in rcccDNA-based mouse model of HBV persistence. (A) C57BL/6 mice transgenic for liver-specific Cre recombinase were injected with Adv/prcccDNA via tail vein, and mice that remained positive for serum HBV antigens (HBsAg and HBeAg) for 4 weeks were injected with 2×10^{11} genome equivalents of AAV-MSX1-Flag ($n = 6$) or control virus AAV-Ctrl ($n = 6$). Sera were collected at indicated time points. At 10 weeks post injection of AAV (w.p.i.), mice were sacrificed, and liver tissues taken. Serum HBsAg (B), HBsAb (C), and HBeAg (D) were assayed using ELISA. Group means and SEMs within group (left panels), as well as group positivity percentage data (right panels) were plotted with group sizes (*n*) indicated. (E) HBV capsid-associated DNA and rcccDNA in liver tissues at 10 w.p.i. were analyzed using Southern blot and PCR, respectively, and capsid levels analyzed using native agarose gel electrophoresis followed by Western blot with serum HBV antigen status indicated. PCR reaction mixtures were subjected to agarose electrophoresis for amplicon size check.

Multiple transcription factors and co-regulators have been shown to modulate EnII/Cp activity (4, 18), and MRE overlaps with the previously reported binding site of C/EBP and upstream half of an HNF4α-binding site (Fig. 5A). C/EBP is a ubiquitous transcription factor that binds "CCAAT" box, while HNF4α is a liver-enriched transcription factor that binds cognate sequences as homodimer, and both are essential for EnII/Cp activity (4, 18). Using reporter assay as well as DNA probe pulldown assay, we demonstrated that overlapping binding sites of MSX1 and HNF4α resulted in competition between the two for MRE binding, which in turn manifested as MSX1-mediated repression that was dependent on the presence of both HNF4α in *trans* and its binding site within MRE in *cis* (Fig. 5 and 6). Recently, a similar mechanism of EnII/Cp repression through competition with HNF4α for DNA binding was reported for another transcription factor MafF (20). On the other hand, host genes SART1 and FoxO4 decrease cccDNA transcription via downregulation of HNF4α expression (24, 25). Collectively, these earlier works and the current report emphasize the importance of HNF4α both as a positive regulator of HBV transcription and as a target for host factor-mediated control of the virus.

Although homeobox domain of MSX1 was crucial for its binding to MRE and repression of EnII/Cp (Fig. 5J through N), the mapped MSX1-binding sequences within MRE (Fig. 6) apparently do not contain the canonical MSX1 cognate motif 5′-C/GTAAT-3′ (26). Details of MSX1-MRE binding, including nucleotide-amino residue interactions and

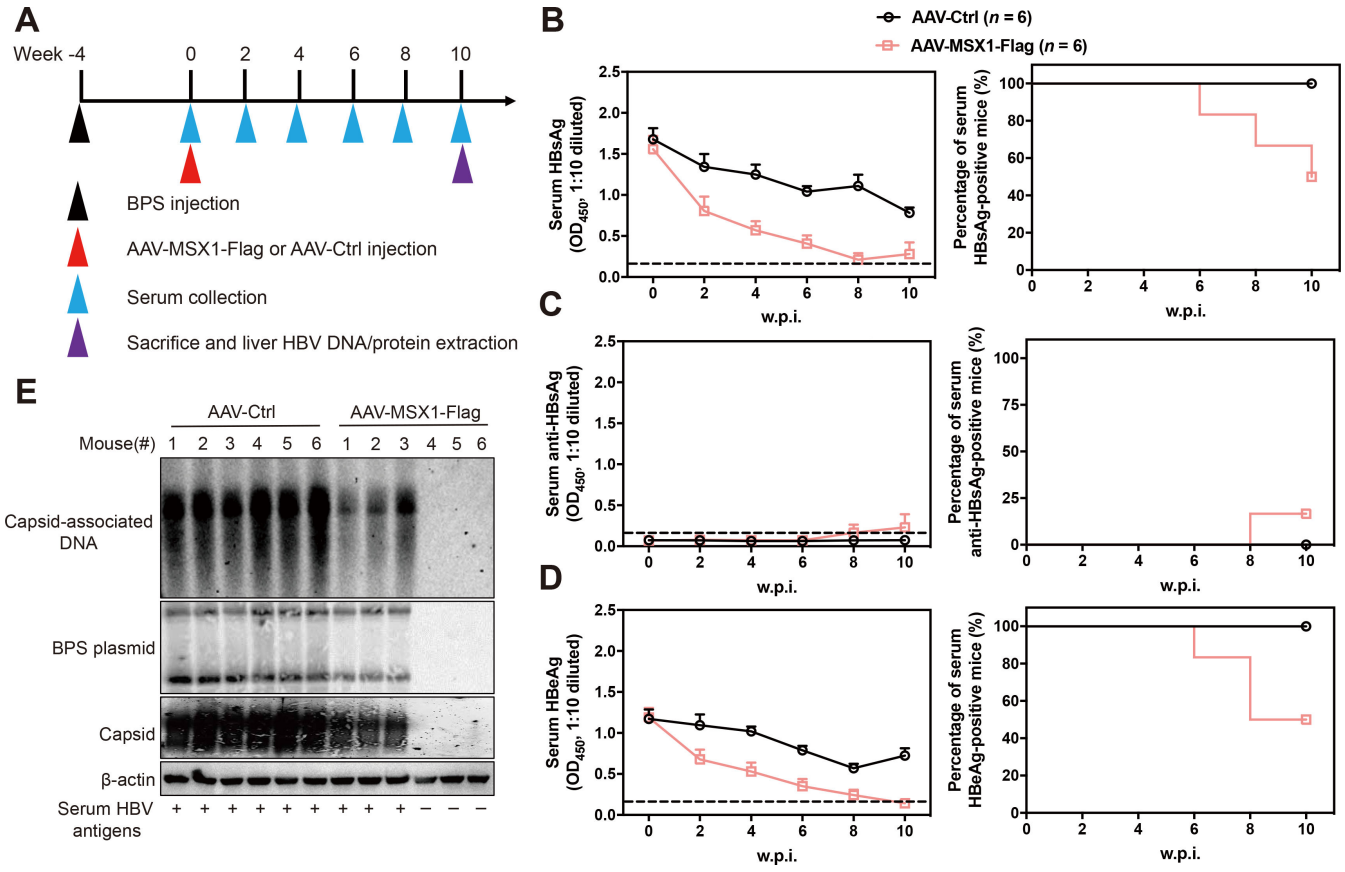

FIG 10 Effects of human MSX1 administration in BPS-based mouse model of HBV persistence. (A) BALB/c mice were injected with 10 µg of BPS replicon plasmids through HDI method via tail vein, and mice that remained positive for serum HBV antigens (HBsAg and HBeAg) for 4 weeks were injected with 2×10^{11} genome equivalents of AAV-MSX1-Flag (n = 6) or AAV-Ctrl (n = 6). Sera were collected at indicated time points. At 10 weeks post injection of AAV (w.p.i.), mice were sacrificed, and liver tissues taken. Serum HBsAg (B), HBsAb (C), and HBeAg (D) were assayed using ELISA. Group means and SEMs within group (left panels), as well as group positivity percentage data (right panels) were plotted with group sizes (n) indicated. (E) HBV capsid-associated DNA and BPS replicon plasmids in liver tissues at 10 w.p.i. were analyzed using Southern blot, and capsid levels analyzed using native agarose gel electrophoresis followed by Western blot with serum HBV antigen status indicated.

possible participating co-factor(s), need to be elucidated through further studies. Moreover, human MSX family contains another member, MSX2, that shares almost identical HO domain sequences with MSX1 but lower similarities in other regions (27). MSX2 also can serve as a transcriptional repressor by binding to regulatory elements through its HO domain (27, 28). Since MSX1 represses EII/Cp through competition with HNF4α for DNA binding via its HO domain, it would be interesting to test whether MSX2 could function in a similar fashion.

By using liver biopsy specimens for immunohistochemistry and Western blot analyses, we confirmed at protein level our previous finding that intrahepatic MSX1 expression was higher in IA phase CHB patients compared to IT phase patients (15). Since HBV gene expression and genome replication tend to be lower in IA phase compared to IT phase (Fig. 7), the possibility that increased EnII/Cp repression by elevated MSX1 expression contributes toward reduced HBV activities in IA phase cannot be ignored, and more in-depth analysis involving longitudinal samples is required to rule out interference caused by differences between patients. The mechanisms leading to elevated MSX1 expression in IA phase also warrant further studies, and the outcome would have significant clinical significance. Interestingly, our recent data using liver biopsy specimen from interferon-α-treated CHB patients showed a more marked increase in MSX1 mRNA in patients with better response to treatment (16), which is also consistent with MSX1-mediated repression as demonstrated here. However, we failed to see MSX1 expression

in primary human hepatocytes increases in response to interferon-α stimulation alone (data not shown). Other immune and non-immune factors with changed expression profiles between IT and IA phases or between interferon-α-responders and non-responders need to be screened to identify upstream regulator(s) of MSX1 in the context of CHB.

The ability of human MSX1 to repress HBV gene expression and genome replication in mouse models (Fig. 9 and 10) provided additional support for its possible regulatory role *in vivo*. More importantly, 33%–50% of AAV-MSX1-Flag-treated mice cleared all forms of HBV DNA from liver by 10 w.p.i. (Fig. 9 and 10). Since the remaining mice in the treated groups also had low serum and intrahepatic HBV markers by 10 w.p.i., higher clearance rates might be possible if observation period was extended. Involvement of anti-hMSX1 immune responses mounted by AAV-MSX1-Flag-injected mice in HBV clearance was unlikely due to the high similarity (~94%) between human and murine MSX1 amino acid sequences. Nevertheless, repeating the experiments using mutant MSX1 deficient in MRE binding (Fig. 5) would rule out this possibility more convincingly while also providing additional correlation with *in vitro* data.

In summary, host homeobox protein MSX1 has been identified as a novel restriction factor of HBV that competes with the essential EnII/Cp positive regulator HNF4α for overlapped binding sites. Changes in MSX1 expression levels during different phases of CHB natural history suggest possible participation in host anti-viral responses, while MSX1-induced viral repression and clearance in mouse models of HBV persistence support it as a potential therapeutic target.

MATERIALS AND METHODS

Cells

Human hepatocellular carcinoma (Huh7 and HepG2), mouse hepatocellular carcinoma (Hepa1-6 and AML12), hepatoma cells with inducible HBV replicon (genotype D) insertion (HepAD38), and HEK293T cell lines were cultured in Dulbecco's modified Eagle medium containing 2 mM L-glutamine, 50 U/mL penicillin, and 10% fetal bovine serum (all from Invitrogen, China). HepG2-NTCP cells (3) were additionally supplemented with 2.5% dimethyl sulfoxide (Sigma, China) and 2 µg/mL puromycin (Invitrogen). PHH cells were cultured as previously described (29).

Plasmid construction, transfection, and reporter assay

Open reading frames (ORFs) of human MSX1 (Gene ID 4487), HNF4α (Gene ID 3172), and MafF (Gene ID 23764) were cloned upstream of Flag tag in pCMV-C-Flag (pCtrl) (Beyotime, China) to obtain plasmids pMSX1-Flag, pHNF4α-Flag, and pMafF-Flag, respectively. His-tagged MSX1 overexpression plasmid (pMSX1-His) was similarly generated. BPS plasmid containing 1.3-fold over-length HBV genome (GenBank Accession Number: AF100309.1) (23), plasmid-carrying precursor of recombinant cccDNA (GenBank Accession Number: V01460.1), and Cre recombinase overexpression plasmid (pCMV-Cre) have been described previously (17).

HBV enhancer/promoter luciferase reporter plasmids have been described previously (30). Briefly, HBV genotype B (GenBank Accession Number: KR232337) EnII/Cp (nt −177 to +36, relative to preC start codon), Sp1 (nt −629 to −68 relative to preS1 start codon), Sp2 (nt −561 to −188 relative to S start codon), and EnI/Xp (nt −424 to +2 relative to HBx start codon) were cloned upstream of firefly luciferase in pGL3-Basic (Promega, China) to produce reporter plasmids pCp, pSp1, pSp2, and pXp, respectively. EnII/Cp 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-EnII, pSp2-EnII, and pXp-EnII, respectively (Fig. 3A). Another EnII/Cp reporter plasmid containing nucleotide sequences spanning nt −399 to +36 (relative to preC start codon) was similarly constructed, and its serial deletion mutant plasmids (Fig. 3D) were generated using site-directed mutagenesis kit (TOYOBO, Japan). Nucleotides were numbered using first nucleotide of respective ORF

as +1 and the immediate upstream nucleotide as −1. Transfection was carried out using TurboFect Transfection Reagent (Thermo, China), and promoter activities were examined using Dual-Luciferase Report Assay System (Promega), according to the manufacturers' instructions.

Viruses and infections

For preparation of lentivirus-mediating MSX1 overexpression, pCDH-MSX1-Flag was generated by inserting Flag-tagged MSX1 downstream of CMV promoter in pCDH (System Biosciences). For preparation of lentivirus-mediating MSX1 knockdown, DNA fragment encoding shMSX1 was cloned downstream of U6 promoter in pLKO.1 (pshCtrl, Addgene USA) to produce pshMSX1. The target sequences of RNA interference were "cccgagaggaccccgtggatgcaga." The plasmids were then co-transfected with helper plasmids psPAX2 and pMD2.G (Addgene, USA) at a ratio of 4:3:1 into HEK293T cells using polyethylenimine (Sigma, China) as previously described (31). 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 infection.

For HBV infection, HBV virions were obtained by concentration of supernatants from HepAD38 cells and then quantified using commercial quantitative assay (Adicon, China). HBV infection of HepG2-NTCP and PHH cells was performed at 1,000:1 multiplicity of infection as previously described (29, 32). At 12 h post infection, cells were washed with 1× phosphate buffer saline (PBS) and changed into fresh media every 1–2 day(s).

Recombinant serotype 8 adeno-associated virus expressing Flag-tagged human MSX1 (AAV-MSX1-Flag) and control virus (AAV-Ctrl) was purchased from Hanbio Technology, China. Recombinant human adenovirus serotype 5 harboring precursor of recombinant cccDNA has been described previously (22).

Western blot

Total cell lysates were prepared from cultured cells, whereas human and mouse liver samples were prepared using SDS lysis buffer (Beyotime, China). Nuclear and cytoplasmic fractions were prepared using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China). Western blot was performed as previously described (33). Antibodies used: anti-Flag (1:10,000) and anti-β-actin (1:10,000) from Sigma; anti-MSX1 (1:3,000, #4787S), anti-HNF4α (1:3,000, C11F12), and anti-His (1:3,000, #2365) from Cell Signaling Technology; anti-Lamin A/C (1:3,000, A0249) and anti-ANXA1 (1:3,000, A1118) from ABclonal; anti-HBc (1:10,000, Denmark) from DAKO. Mouse anti-HBx mAb 2A7 (1:1,000) has been previously described (34, 35) .

HBV antigens and nucleic acids analysis

HBsAg, HBeAg, and HBsAb were measured using ELISA Kits (Kehua, China). Total cellular RNA, HBV capsid-associated DNA and nuclear DNA, and cccDNA-containing extrachromosomal nuclear DNA were extracted as previously described (23). For Northern blot, 10 µg of RNA was subjected to electrophoresis in 1.5% agarose containing 2.2 mol/L formaldehyde and transferred onto positively charged nylon membrane (Roche, Germany). For Southern blot, DNA samples were subjected to electrophoresis in 1% agarose and transferred onto nylon membrane. Both membranes were hybridized with DIG-labeled full-length HBV probe, and signals developed using DIG Luminescent Detection Kit (Roche).

Quantitative real-time PCR for mRNA and HBV cccDNA

For mRNA quantification, total cellular RNA was subjected to reverse transcription using PrimeScriptRT Reagent Kit (TaKaRa, China) to generate cDNA. cDNA and HBV cccDNA were quantified in real-time PCR using SYBR Premix Ex Taq II Kit (TaKaRa). Primers used:

3.5 kb RNA forward primer 5′-AATGCCCCTATCTTATCAACACT-3′ and reverse primer 5′-GAGATTGAGATCTTCTGCGACG-3′, β-actin forward primer 5′-AAGGTGACAGCAGTCGGTT-3′ and reverse primer 5′- TGTGTGGACTTGGGAGAGG-3′, cccDNA forward primer 5′- TGCACTTCGCTTCACCT-3′ and reverse primer 5′-AGGGGCATTTGGTGGTC-3′.

Immunofluorescence and ChIP

Immunofluorescence assay performed on cultured cells was described previously (36) . Briefly, cells were firstly washed with PBS, fixed in 4% paraformaldehyde for 30 min, and washed with PBS. The cells were blocked with 3% BSA in PBST (PBS plus 0.5% Triton X-100) for 30 min, stained with primary antibody against Flag or HBc (1:1,000, as listed for Western blot) or MSX1 (1:1,000, #A18295, ABclonal) for 2 h at room temperature, and finally incubated with Alexa Fluor 546- or 488-labeled secondary antibodies (1:1,000, Life Technologies, USA) for 1 h at room temperature. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (Sangon, China) and observed using an AMG EVOS fluorescence microscope (Mill Creek, USA).

ChIP assay was performed using SimpleChIP Plus Enzymatic Chromatin IP Kit (#9005, Cell Signaling Technology) according to the manufacturer's instruction. MSX1 and Flag antibodies as listed above for Western blot were used.

Cell viability and *in vitro* protein translation

Cell viability was assessed using a Cell Counting Kit-8 (CCK8) (Dojindo, Japan), and *in vitro* translated MSX1 was prepared using TnT T7 Quick Coupled Transcription/Translation System (Promega), according to the manufacturers' instructions.

DNA pulldown and Co-IP assays

For DNA pulldown assay, nuclear extracts from Huh7 cells or *in vitro* translated MSX1 proteins were mixed with streptavidin-coupled Dynabeads (Thermo, China) and biotinylated double-stranded DNA probes (Genwiz, China) in the binding solution buffer (Beyotime, China). After incubation with rotation at 4°C for 6 h, beads were washed five times with PBST (PBS plus 0.1% Tween-20) and then heated in SDS lysis at 100°C for 10 min, and the supernatants were subjected to Western blot.

Co-IP assay has been described previously (31, 37) using the antibodies as listed above for Western blot. Anti-FLAG Magnetic Beads (M8823) were purchased from Sigma.

Patient samples and immunohistochemistry analysis

We previously enrolled a total of 58 CHB patients, with 22 patients in IT phase and 36 patients in IA phase (15). Liver biopsy was performed on all patients, and samples were subjected to microarray analysis using Affymetrix Human Genome U133 Plus 2.0 Array in our previous studies (15). Biopsy samples from 20 IT and IA patients (Table 1) were assayed for MSX1 protein expression. Intrahepatic expression of MSX1 and HBc was examined by performing immunohistochemistry analysis on paraffin-fixed liver biopsy specimen using antibodies against MSX1 (1:1,000, #A18295, ABclonal) and HBc (1:500, as listed above for Western blot). Relative protein expression levels were presented using H-SCORE as calculated by Caseviewer 2.0 on images captured by Pannoramic P250 (3DHISTECH, Hungary).

Animal experiments

Male BALB/c and C57BL/6 mice aged 6–8 weeks were purchased from Shanghai Slac Laboratory Animal Co. Ltd. Male 6- to 8-week-old C57BL/6 mice transgenic for liver-specific Cre recombinase (Cre Tg C57BL/6) have been described previously (22). For determining the effects of human MSX1 on Cp activity and HBx expression *in vivo*, C57BL/6 mice were injected with 2×10^{11} genome equivalents (geq) of AAV-MSX1-Flag or its control virus (AAV-Ctrl) in 200 µL in PBS via tail vein. At day 7 post injection of

AAV, mice were injected with 2 µg of pCp and 0.5 µg of pRL-TK through hydrodynamic injection via tail vein. At day 2 post injection of plasmids, mice were sacrificed by carbon dioxide treatment to collect liver tissues. Cp activity and exogenous MSX1 expression in livers were measured using dual-luciferase reporter assay and Western blot, respectively.

For obtaining rcccDNA-based persistence mice, Cre Tg C57BL/6 mice were injected with 1.5×10^9 geq of Adv/prcccDNA in 200 µL PBS via tail vein (22). For obtaining BPS-based persistence mice, BALB/c mice were injected with 10 µg of BPS replicon plasmids via HDI method (23). Mice that remained positive for serum HBV antigens for 4 weeks post injection of Adv/prcccDNA or BPS were taken as harboring rcccDNA or BPS persistence. To investigate whether human MSX1 harbors any therapeutic effects against CHB, HBV persistence mice were injected with 2×10^{11} geq of AAV-MSX1-Flag or AAV-Ctrl. Mouse serum samples and liver tissues were collected through retro-orbital sinus bleeding and carbon dioxide treatment, respectively, at indicated time points. PCR analysis was performed to examine the rcccDNA and GAPDH DNA in hepatocytes. The primers used: rcccDNA forward primer 5′-CAAGACAGGTTTAAGGAGAC-3′ and reverse primer 5′-CTGCGGTATTGTGAGGATTC-3′, GAPDH forward primer 5′- TGCCCAGAACATCATC CCTG-3′ and reverse primer 5′- TCAGATCCACGACGGACACA -3′.

Statistical analysis

Group means and standard errors from independently repeated experiments were indicated and subjected to unpaired two-tailed *t*-test. Group positivity percentages of serum HBV antigens were presented using Kaplan-Meier plot. A *P*-value <0.05 was considered statistically significant. GraphPad 6 was used for plotting and statistical tests.

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AUTHOR AFFILIATIONS

[1]Department of Infectious Diseases, Shanghai Institute of Infectious Diseases and Biosecurity, Shanghai Key Laboratory of Infectious Diseases and Biosafety Emergency Response, National Medical Center for Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China
[2]Key Laboratory of Medical Molecular Virology (Ministry of Education/National Health Commission/Chinese Academy of Medical Sciences), Shanghai Frontiers Science Center of Pathogenic Microbes and Infection, Department of Microbiology and Parasitology, School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Shanghai, China
[3]Department of Infectious Diseases, First Hospital of Quanzhou Affiliated to Fujian Medical University, Quanzhou, China
[4]Shanghai Key Laboratory of Medical Epigenetics, Institutes of Biomedical Sciences, Shanghai Medical College, Fudan University, Shanghai, China
[5]Children's Hospital, Fudan University, Shanghai, China

AUTHOR ORCIDs

Zhongliang Shen http://orcid.org/0000-0001-8566-9149
Jing Liu http://orcid.org/0000-0002-9728-8893
Youhua Xie http://orcid.org/0000-0002-2416-7708

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AUTHOR CONTRIBUTIONS

Zhongliang Shen, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – original draft, Writing – review and editing | Shenyan Zhang, Conceptualization, Data curation, Investigation, Project administration, Software | Zixiang Gao, Data curation, Project administration, Software, Visualization | Xueping Yu, Investigation, Project administration, Resources | Jinyu Wang, Data curation, Formal analysis, Investigation, Project administration | Shaokun Pan, Methodology, Resources, Software | Ning Kang, Methodology, Resources, Software | Nannan Liu, Formal analysis, Resources, Software | Huijun Xu, Data curation, Resources, Software | Mu Liu, Methodology, Resources, Software | Yang Yang, Resources, Software | Qiang Deng, Formal analysis, Resources | Jing Liu, Conceptualization, Supervision, Visualization, Writing – review and editing | Youhua Xie, Conceptualization, Funding acquisition, Supervision, Writing – review and editing | Jiming Zhang, Conceptualization, Funding acquisition, Investigation, Supervision

ETHICS APPROVAL

The study received permission from the Ethics Committee of Huashan Hospital (KY2021-920). Written informed consent was obtained from all enrollees. Mouse procedures were approved by the Animal Ethics Committee of School of Basic Medical Sciences, Fudan University (2021JS-063).

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