RESEARCH ARTICLE

Upregulation of MicroRNA-146a by Hepatitis B Virus X Protein Contributes to Hepatitis Development by Downregulating Complement Factor H

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ABSTRACT **Hepatic injuries in hepatitis B virus (HBV) patients are caused by immune responses of the host. In our previous study, microRNA-146a (miR-146a), an innate immunity-related miRNA, and complement factor H (CFH), an important negative regulator of the alternative pathway of complement activation, were differentially expressed in HBV-expressing and HBV-free hepatocytes. Here, the roles of these factors in HBV-related liver inflammation were analyzed in detail. The expression levels of miR-146a and CFH in HBV-expressing hepatocytes were assessed via analyses of hepatocyte cell lines, transgenic mice, adenovirus-infected mice, and HBV-positive human liver samples. The expression level of miR-146a was upregulated in HBV-expressing Huh-7 hepatocytes, HBV-expressing mice, and patients with HBV infection. Further results demonstrated that the HBV X protein (HBx) was responsible for its effects on miR-146a expression through NF-κB-mediated enhancement of miR-146a promoter activity. HBV/HBx also downregulated the expression of *CFH* mRNA in hepatocyte cell lines and the livers of humans and transgenic mice. Furthermore, overexpression and inhibition of miR-146a in Huh-7 cells downregulated and upregulated *CFH* mRNA levels, respectively. Luciferase reporter assays demonstrated that miR-146a downregulated *CFH* mRNA expression in hepatocytes via 3′-untranslated-region (UTR) pairing. The overall effect of this process *in vivo* is to promote liver inflammation. These results demonstrate that the HBx–miR-146a–CFH–complement activation regulation pathway might play an important role in the immunopathogenesis of chronic HBV infection. These findings have important implications for understanding the immunopathogenesis of chronic hepatitis B and developing effective therapeutic interventions.**

IMPORTANCE **Hepatitis B virus (HBV) remains an important pathogen and can cause severe liver diseases, including hepatitis, liver cirrhosis, and hepatocellular carcinoma. Although HBV was found in 1966, the molecular mechanisms of pathogenesis are still poorly understood. In the present study, we found that the HBV X protein (HBx) promoted the expression of miR-146a, an innate immunity-related miRNA, through the NF-κB signal pathway and that increasingly expressed miR-146a downregulated its target complement factor H (CFH), an important negative regulator of the complement alternative pathway, leading to the promotion of liver inflammation. We demonstrated that the HBx–miR-146a–CFH–complement activation regulation pathway is potentially an important mechanism of immunopathogenesis caused by chronic HBV infection. Our data provide a novel molecular mechanism of HBV pathogenesis and thus help to understand the correlations between the complement system, an important part of innate immunity, and HBV-associated disease. These findings will also be important to identify potential therapeutic targets for HBV infection.**

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Hepatitis B virus (HBV) infection is a global public health problem that affects more than 400 million people worldwide (1). HBV infects hepatocytes but is not directly cytopathic; instead, the resulting hepatic injuries are believed to be caused by immune responses of the host. The immunopathogenesis of hepatitis B depends on a complex interplay of host factors, such as age, gender, and immune status. More than 50% of people with chronic hepatitis B (CHB) are lifetime asymptomatic, whereas 15 to 40% develop liver cirrhosis and hepatocellular carcinoma (HCC) (2), which has been attributed to repeated immune responses characterized by continuous cycles of low-level liver cell destruction and regeneration (3). Although great effort has been invested in understanding the molecular mechanisms that determine HBV pathogenesis, some major questions remain unanswered (1, 3, 4).

One of the major objectives of the CHB research community is to identify the molecular determinants of CHB progression that could facilitate prognosis and management of the disease. A range

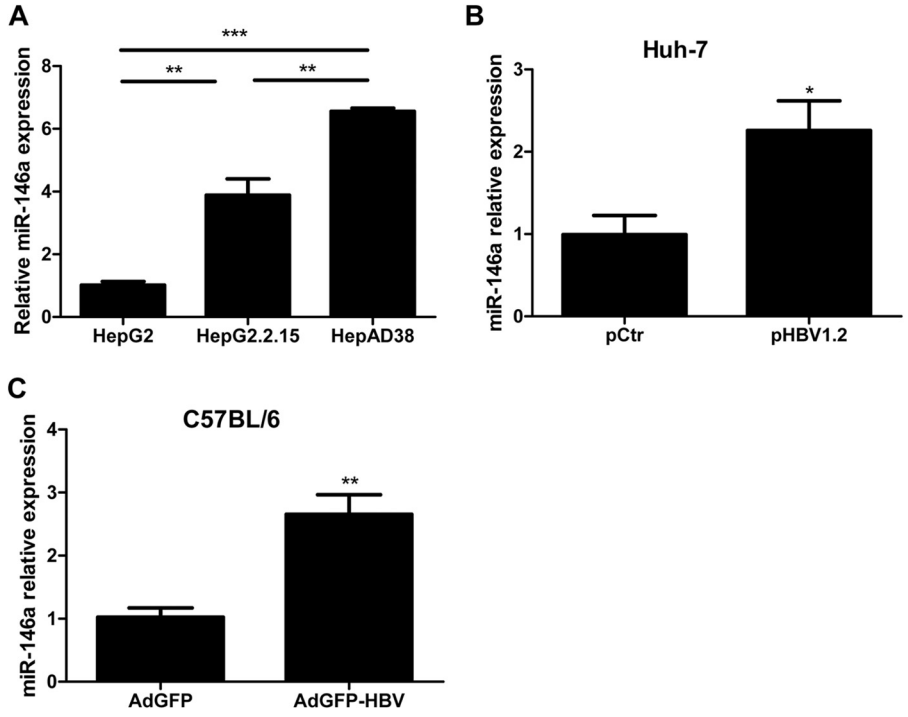

FIG 1 miR-146a is upregulated in HBV-replicating hepatocytes and livers of HBV-infected mice. (A) Quantitative RT-PCR analyses of the relative expression levels of endogenous miR-146a in HepG2, HepG2.2.15, and HepAD38 cells. Huh-7 cells transiently transfected with pHBV1.2 or a control vector (pCtr) (B) and liver tissues of C57BL/6 mice 7 days after receiving a hydrodynamic injection of AdGFP-HBV or AdGFP (C). Each assay was performed in triplicate, and the expression level of miR-146a was normalized to that of U6 snRNA. Data are presented as the means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus control or as indicated.

of host molecules have been studied, including cytokines, chemokines, complements, and, in recent years, microRNAs (miRNAs). miRNAs are short (approximately 22 nucleotides), endogenously expressed, noncoding RNAs that regulate gene expression at the posttranscriptional level by pairing with the 3′ untranslated regions (UTRs) of target transcripts, leading to translational inhibition and/or mRNA degradation (5). In fact, miRNAs represent a universal regulatory mechanism (6).

Several groups have studied the roles of miRNAs in HBV pathogenesis (7). We previously have demonstrated that miRNA-15b modulates HBV replication through targeting hepatocyte nuclear factor 1α (8). To identify the key molecules involved in HBV-induced hepatitis, we systematically analyzed the miRNA and mRNA expression profiles of HepG2, HepG2.2.15 (a stable cell line with low HBV replication), and HepAd38 (a stable cell line with higher inducible HBV replication than HepG2.2.15) cells (8). We found that the miR-146a expression level was positively correlated with the HBV replication level. miR-146A modulates both the innate and adaptive immune responses via negative feedback loops involving downregulation of its target genes (9). It could target tumor necrosis factor (TNF) receptor-associated factor 6 and other key effectors of various Toll-like receptor (TLR) signaling pathways (10); however, different cells and disease conditions, such as various tumors (11), rheumatoid arthritis (12), and stressed neural cells (13), are associated with the use of different miR-146a effectors.

It has been reported that the plasma levels of complement component 3 (C3) and C4 are significantly lower in more severe CHB cases than in normal or mild CHB cases (14, 15). Our laboratory has been studying the involvement of complements in the immunopathogenesis of CHB for a number of years. Based on our analysis of the mRNA expression profiles in HBV-expressing hepatocytes and prediction of the potential targets of miR-146a *in silico*, complement factor H (CFH) was selected for further study. CFH is a major inhibitor of the alternative pathway of complement activation (16). To examine their possible mechanisms of regulation and impact on liver inflammation during HBV infection, the hepatic expression levels of miR-146a and CFH were determined both *in vitro* and *in vivo*. Our results reveal an inverse relationship between the expression levels of miR-146a and CFH and demonstrate that both of them play important roles in the pathogenesis of HBV-induced hepatitis.

RESULTS

miR-146a is upregulated in HBV-replicating hepatocytes and hepatic tissues of HBV-expressing mice. In our previous study, we used microarrays to analyze the miRNA expression profiles of HBV-replicating (HepG2.2.15 and HepAd38 cells) and HBV-free (HepG2) hepatocytes (8) and demonstrated that the expression level of miR-146a is positively correlated with the level of HBV replication. The finding was confirmed by quantitative reverse transcription-PCR (qRT-PCR) (Fig. 1A). Furthermore, an HCC cell line (Huh-7) transfected with a vector containing a 1.2-fold HBV genome (pHBV1.2) had a higher level of miR-146a than cells transfected with a control vector (Fig. 1B), suggesting that HBV induces overexpression of miR-146a directly. Next, we performed hydrodynamic injection of an adenovirus containing green fluorescent protein (AdGFP)-HBV or AdGFP into C56BL/6 mice and

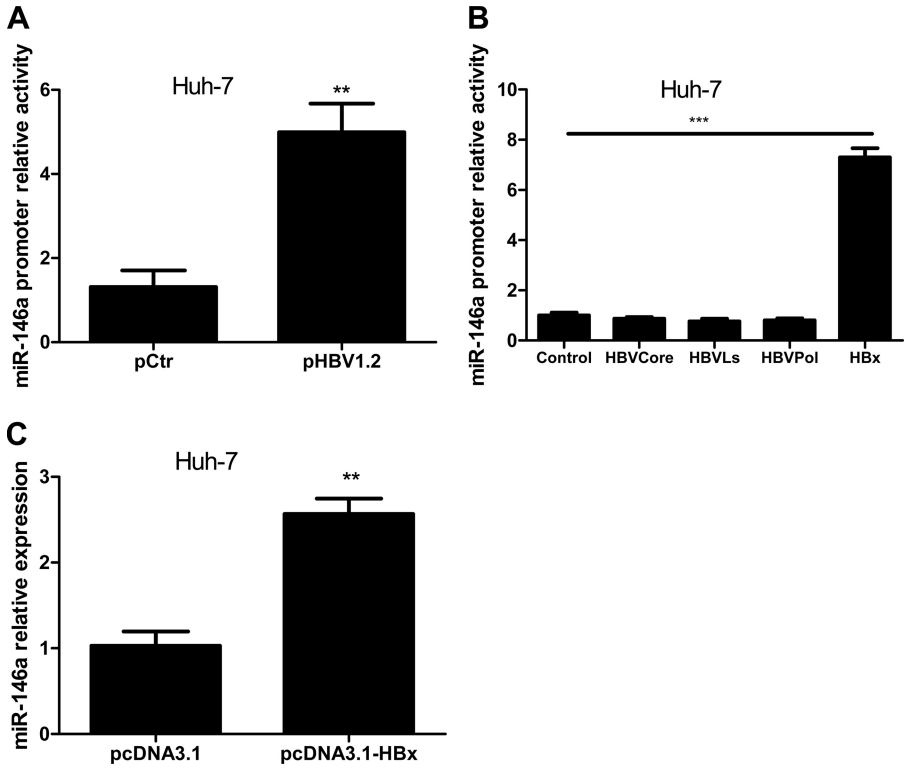

FIG 2 HBx induces miR-146a expression by enhancing its promoter activity. (A and B) The activity of a luciferase reporter gene under the control of the miR-146a promoter in cells that were cotransfected with pGL-146aP and pHBV1.2 (A) or a pcDNA3.1-based vector expressing HBc (HBVCore), large surface protein (HBVLs), viral polymerase (HBVPol), or HBx (B). (C) Quantitative RT-PCR analyses of miR-146a expression in Huh-7 cells that were transiently transfected with pcDNA3.1-HBx or empty vector. The expression level of miR-146a was normalized to that of U6 snRNA. Data are presented as the means ± SEM from $n = 3$ replicates. **, $P < 0.01$; ***, $P < 0.001$, versus control.

found that miR-146a expression in mouse liver was significantly higher in the AdGFP-HBV group than in the AdGFP group (Fig. 1C). Taken together, these data indicate that HBV infection of hepatocytes enhances miR-146a expression significantly.

HBV X protein induces miR-146a expression by enhancing its promoter activity. To determine whether HBV enhances miR-146a expression by acting on its promoter, Huh-7 cells were cotransfected with a luciferase reporter plasmid containing the miR-146a promoter (pGL3-146aP) and pHBV1.2. As shown in Fig. 2A, HBV expression enhanced the activity of the miR-146 promoter. To identify which HBV component caused this effect, Huh-7 cells were cotransfected with pGL3-146aP and a pcDNA3.1 vector containing the gene encoding the HBV core protein (HBc), large surface protein, viral polymerase, or X protein (HBx). Overexpression of HBx enhanced the activity of the miR-146 promoter, but overexpression of the other proteins had no effect (Fig. 2B). Furthermore, a qRT-PCR analysis revealed that the expression level of miR-146a was higher in Huh-7 cells transfected with pcDNA3.1-HBx than in those transfected with empty pcDNA3.1 vector (Fig. 2C). Overall, these results suggest that HBV promotes miR-146a expression through upregulation of miR-146a promoter activity by HBx.

The NF-κB signaling pathway mediates HBx-induced upregulation of miR-146a. To study the mechanism of HBx-mediated upregulation of miR-146a promoter activity, we generated luciferase reporter plasmids containing the 5′ (pGL3-146apS1) and 3′ (pGL3-146apS2) segments of the miR-146a promoter and tested their activities in Huh-7 cells. The reporter activity of pGL3-146apS1 was minimal, while the activity of pGL3-146apS2 was similar to that of the full-length miR146a promoter (Fig. 3A). In addition, overexpression of HBx by cotransfection with pcDNA3.1-HBx enhanced the activities of the full-length and 3′ segment of the miR-146a promoter significantly but had no effect on that of the 5′ segment (Fig. 3B). Scanning of the 3′ segment of the miR-146a promoter (nucleotides −481 to +21 relative to the start of transcription) identified two NF-κB binding sites (Fig. 3A), as described previously (17). HBx reportedly regulates NF-κB activity (18); therefore, we hypothesized that HBx promotes binding of NF-κB to its consensus sites in the miR-146a promoter, thereby upregulating the promoter activity. Mutation of the two NF-κB binding sites in the 3′ segment of the miR-146a promoter (Fig. 3A) abolished the upregulation effect on miR-146a promoter activity of HBx (Fig. 3B). Furthermore, treatment of transfected Huh-7 cells with pyrrolidine dithiocarbamate, an inhibitor of NF-κB signaling, inhibited the HBx-mediated enhancement of miR-146a promoter activity (Fig. 3C) and upregulation of miR-146a expression (Fig. 3D). These experiments provide further evidence that HBx mediates upregulation of miR-146a via the NF-κB signaling pathway.

HBx downregulates *CFH* expression in hepatocytes and hepatic tissue. As mentioned earlier, in a previous study we systematically analyzed the mRNA expression profiles of HBV-expressing (HepAD38 and HepG2.2.15) and HBV-free (HepG2) hepatocytes (unpublished data). Here, *CFH*, an important nega-

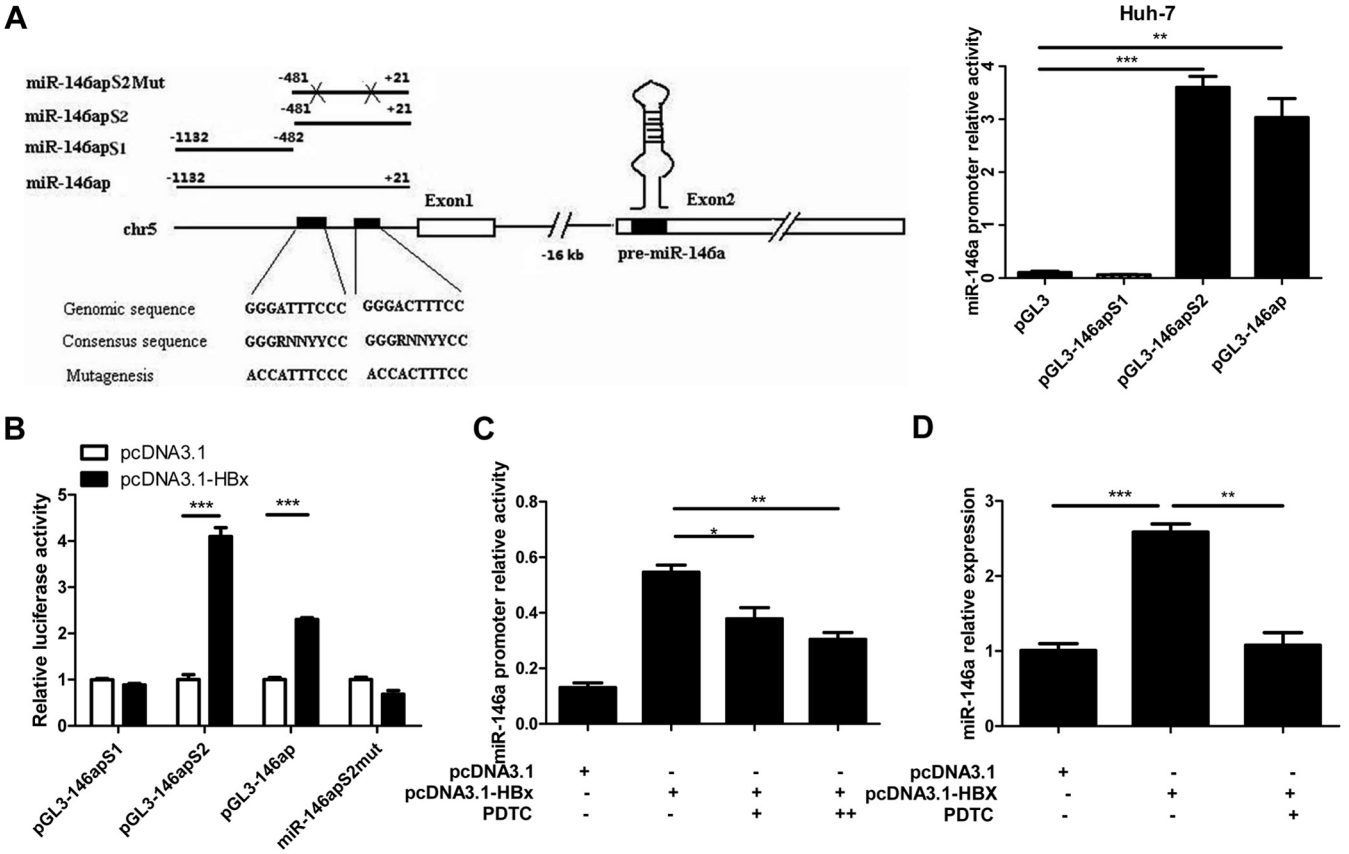

FIG 3 HBx upregulates the activity of the miR-146a promoter through NF-κB. (A) The left panel shows a schematic illustration of the miR-146a promoter segments used to generate the PGL3 luciferase reporter constructs. The wild-type and mutated NF-κB binding sites are also shown. The right panel shows the luciferase activities of the PGL3 reporter constructs containing the miR-146a promoter regions. The assays were performed 48 h after transfection of Huh-7 cells. (B) Effect of transient overexpression of HBx on the activities of the miR-146a promoter reporter constructs shown in panel A; (C and D) effects of pyrrolidine dithiocarbamate (PDTC), an NF-κB signaling inhibitor, on the HBx-mediated increases in miR-146a promoter activity (C) and miR-146a expression (D) in transiently transfected Huh-7 cells. Data are presented as the means ± SEM from $n = 3$ replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus control or as indicated.

tive regulator of the alternative pathway of complement activation that was identified as an mRNA whose expression showed an inverse relationship with HBV replication level, was selected for further analysis. This finding was confirmed by real-time PCR (Fig. 4A), and the expression level of *CFH* mRNA was significantly lower in Huh-7 cells transfected with pHBV1.2 (Fig. 4B) or pcDNA3.1-HBx (Fig. 4C) than in Huh-7 cells transfected with control plasmids. Finally, *CFH* mRNA and protein levels were significantly lower in the liver tissues of 7- or 16-month-old HBx transgenic mice than those of 7-month-old wild-type mice (Fig. 4D and E).

miR-146a mediates HBx-induced downregulation of *CFH* expression. To determine whether the inhibition of *CFH* expression by HBx occurs via an effect on the *CFH* promoter, we constructed a pGL3 reporter plasmid containing the luciferase gene under the control of the *CFH* promoter and cotransfected Huh-7 cells with this plasmid and pcDNA3-HBx. In these experiments, overexpression of HBx had no effect on the activity of the *CFH* promoter (data not shown); therefore, we hypothesized that HBx-mediated downregulation of *CFH* expression occurs via mRNA degradation. To test this proposal, the 3′ UTR of *CFH* or its miR-146a binding site mutant was cloned into pMIR-luciferase, a vector used to identify miRNA targets, to generate pMIR-CFH3′ UTR. In cotransfected Huh-7 cells, overexpression of HBx inhibited the reporter activity of pMIR-CFH3′ UTR but did not affect that of the control vector (Fig. 5A), suggesting that HBx acts on the 3′ UTR of *CFH* in hepatocytes, possibly via an miRNA-mediated mechanism. In fact, it has been reported previously that miR-146a acts directly on the 3′ UTR of *CFH* in human neural cells to inhibit *CFH* mRNA and protein expression (19).

Transient overexpression of miR-146a in Huh-7 cells downregulated *CFH* mRNA and protein expression (Fig. 5B and C) and the reporter activity of pMIR-CFH3′ UTR (Fig. 5D) significantly but did not influence the reporter activity of the mutant (pMIR-CFH3′ UTR-M) (Fig. 5D). In contrast, transfection of Huh-7 cells with an miR-146a antisense RNA vector (pSD14–miR-146a) resulted in a dramatic increase in the reporter activity of pMIR-CFH3′ UTR (Fig. 5D). The inhibition effect of HBx on the reporter activity of pMIR-CFH3′ UTR was also abrogated when the miR-146a binding site of the *CFH* 3′ UTR was mutated (Fig. 5E). Overexpression of HBx in Huh-7 cells also suppressed the reporter activity of pMIR-CFH3′ UTR, and coexpression of pSD14–miR-146a abrogated this effect (Fig. 5F). These experi-

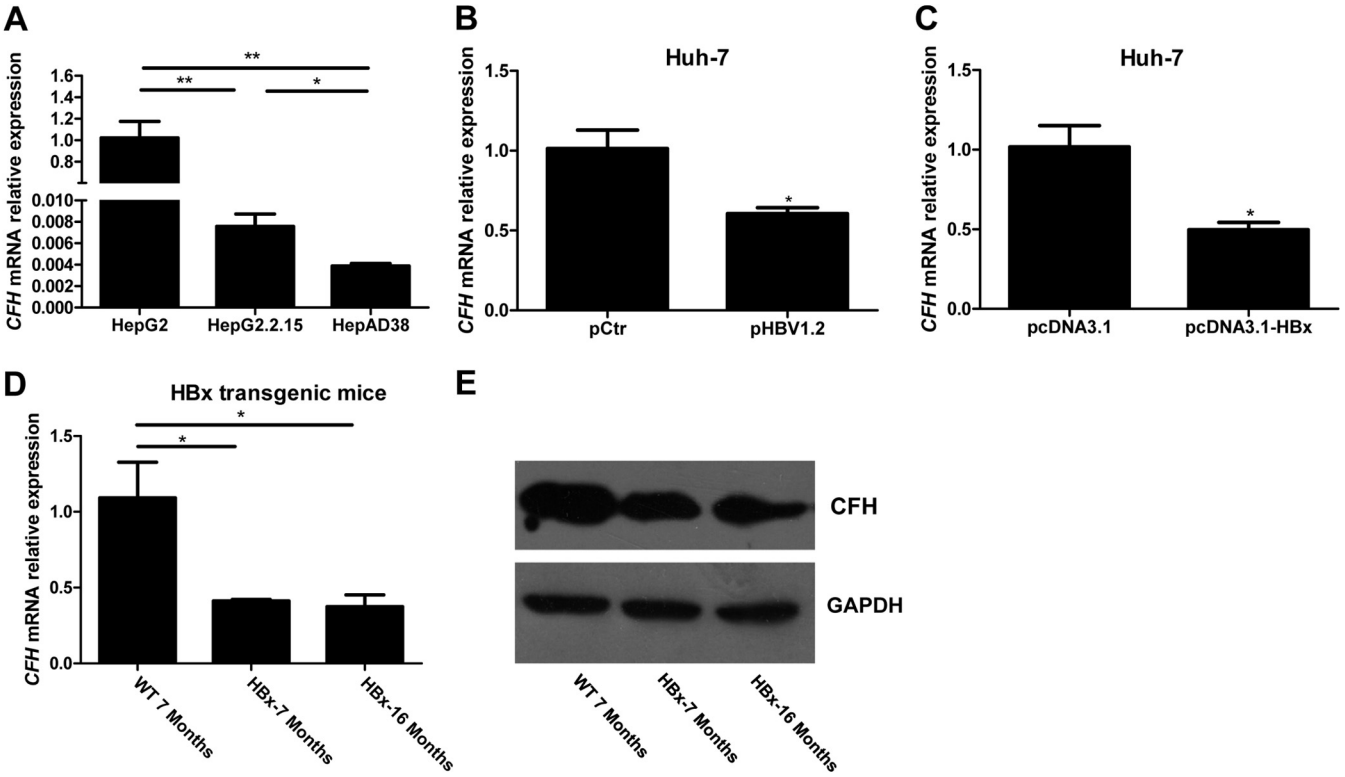

FIG 4 HBx downregulates *CFH* expression in hepatocytes and hepatic tissues. (A to D) Quantitative RT-PCR analyses of *CFH* mRNA levels in HepG2, HepG2.2.15, and HepAD38 cells (A), Huh-7 cells that were transiently transfected with a control vector, pHBV1.2 (B) or pcDNA3.1-HBx (C), and liver tissues of 7-month-old wild-type (WT) mice and 7- and 16-month-old p21-HBx-transgenic mice (D). (E) Immunoblot analyses of CFH protein in liver tissues of WT and p21-HBx-transgenic mice. The expression level of GAPDH was used as the internal control. Data are presented as the means ± SEM from $n = 3$ replicates. *, $P < 0.05$; **, $P < 0.01$, versus control (A to C) or WT (D) mice.

ments indicate that suppression of *CFH* expression by HBx occurs through an miR-146a-mediated effect.

Reduced *CFH* expression is associated with HBV/HBx-induced liver inflammation. HBV can cause acute or chronic inflammation of the human liver, and mice can also present liver damage and inflammation (20–22). Hydrodynamic transfection of normal C57BL/6 mice with a recombinant adenovirus carrying the HBV genome or HBx, AdGFP-HBV or AdGFP-HBx, resulted in decreased hepatic levels of *CFH* mRNA (Fig. 6A) and protein (Fig. 6B). The sequence of mouse mature miR-146a is the same as that of human, and the 3′ UTR of mouse *CFH* also has miR-146a binding sites. Together with the results described above, these findings supported that HBV/HBx causes downregulation of *CFH* expression through upregulation of miR-146a in a mouse model.

The serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) were significantly higher in the AdGFP-HBV- and AdGFP-HBx-infected C57BL/6 mice than in those infected with AdGFP only (Fig. 6C). A histological examination also revealed marked inflammatory responses in mice infected with AdGFP-HBV or AdGFP-HBx (Fig. 6D). In contrast, C3-deficient mice infected with AdGFP-HBV showed a subdued inflammatory response in the liver, despite some localized necrosis (Fig. 6D). Combined with the finding that HBV/HBx expression in mice causes downregulation of *CFH*, these results suggest that the complement system is the effector of the inflammatory response in HBV-related hepatitis.

Cirrhosis is the advanced stage of hepatitis. To support our results obtained using hepatic cell lines and mouse models, we examined the expression levels of miR-146a and *CFH* mRNA in liver samples from human liver transplant patients who were diagnosed with HBV-related cirrhosis ($n = 9$). As a control, samples from normal (HBV-negative) liver transplant donors ($n = 8$) were also examined. The expression level of miR-146a was significantly higher in HBV-related cirrhosis patients than in normal controls (Fig. 7A). In contrast, the *CFH* mRNA expression level was significantly lower in HBV-related cirrhosis patients than in normal controls (Fig. 7B). These data from human patients are consistent with the observations made in mouse models, indicating that HBV-related liver inflammation is associated with enhanced expression of miR-146a and decreased expression of *CFH*.

DISCUSSION

The findings presented here reveal the underlying molecular mechanism by which HBx, the major regulatory protein of HBV, downregulates complement factor H, a key host inhibitory modulator of the alternative pathway of complement activation. The results described above support a regulatory cascade in which (i) HBx enhances miR-146a promoter activity through an NF-κB-mediated effect and (ii) HBx-induced miR-146a downregulates *CFH* by targeting its 3′ UTR. The resulting reduced inhibition of complement activation by CFH would lead to tissue inflammation. Thus, we propose that the HBx–miR-146a–CFH–complement regulation axis may underlie chronic HBV infection-related immunopathogenesis (Fig. 8).

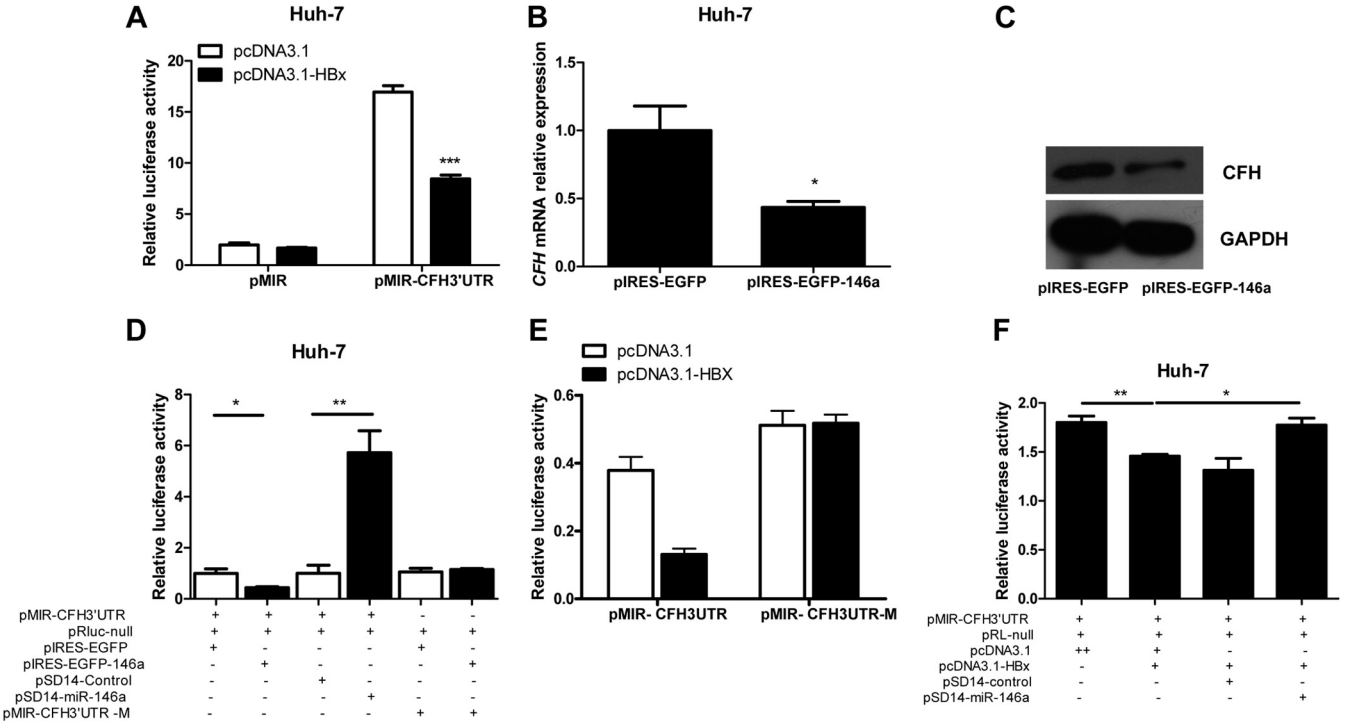

FIG 5 HBx-mediated downregulation of *CFH* expression requires miR-146a. (A) The effect of overexpression of HBx on the activity of a reporter gene under the control of the *CFH* 3' UTR. Dual-luciferase reporter assays of Huh-7 cells that were cotransfected with pcDNA3.1 or pcDNA3.1-HBx and pMIR or pMIR-CFH3'UTR were performed 48 h after transfection. Quantitative RT-PCR (B) and immunoblot analyses (C) of the effect of transient overexpression of miR-146a (pIRES-EGFP-146a) on *CFH* mRNA levels in Huh-7 cells. The effects of overexpression (pIRES-EGFP-146a) (C) or inhibition (pSD14-146a) (D) of miR-146a, or overexpression of HBx (pcDNA3.1-HBx) with or without inhibition of miR-146a (E and F), on the activity of pMIR-CFH3' UTR or pMIR-CFH3' UTR-M in transiently cotransfected Huh-7 cells. The reporter activities were determined at 48 h posttransfection. (A to D) Data are presented as the means ± SEM from $n = 3$ replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To our knowledge, the molecular details of NF-κB-dependent upregulation of miR-146a by HBx and downregulation of *CFH* by miR-146a in hepatocytes are reported here for the first time. Previous studies have demonstrated that NF-κB-dependent activation of miR-146a is involved in the regulation of factors such as lipopolysaccharide (17) and interleukin 1β (23) and also occurs during vesicular stomatitis virus (24), human T-cell lymphotropic virus 1 (25), and Epstein-Barr virus (26, 27) infections. The results presented here demonstrate that the NF-κB signaling pathway mediates HBV/HBx-induced upregulation of miR-146a. Downregulation of *CFH* by miR-146a has been reported previously in human neural cells (13, 19); the results presented here demonstrate that this regulation also occurs in hepatocytes. HBx reportedly induces NF-κB activation in hepatocytes through physical interaction with p22-FLIP and NEMO (18); in combination with our observations, this finding presents the complete molecular picture of HBx-mediated upregulation of miR-146a and downregulation of *CFH* in hepatocytes.

miR-146a has emerged as a master regulator of the immune system and is involved in innate and adaptive immunity, various viral infections, cancers, and some nonimmune human diseases (9–11). Multiple targets of miR-146a have been identified to date (9). A most notable example is that in lipopolysaccharide-induced cross-tolerance, miR-146a acts as a central tuning mechanism to prevent an overstimulated acute inflammatory response (28). In HBV-related studies, miR-146a was reported to suppress the sensitivity of HCC cells to interferon alpha through SMAD4 (29), and the CC genotype of the miR-146a rs2910164 G/C polymorphism is associated with susceptibility for acute-on-chronic hepatitis B liver failure (30), while the GG genotype is associated with higher expression of miR-146a and higher risk of HCC, especially in Asian and male populations (31, 32). It is possible that the higher miR-146a expression level found in individuals with the GG genotype leads to enhanced suppression of the acute immune responses that involve activation of the NF-κB pathway, resulting in reduced susceptibility to acute liver failure. However, the results presented here suggest that higher miR-146a expression leads to a more severe suppression of CFH in hepatocytes, resulting in enhanced chronic complement-mediated cytotoxicity and subsequent liver fibrosis, cirrhosis, and HCC. Therefore, miR-146a appears to be a key molecule that has the potential to influence the direction of progressive HBV infection; as such, further investigation of the roles of miR-146a expression in HBV-related innate immunity and tumorigenesis is warranted.

The pathogenesis of HBV-induced hepatitis is complicated. The complement system, which plays an important role in various inflammatory conditions, reportedly plays a pivotal role in chemical-induced hepatitis (33) and liver-specific autoantibody-mediated hepatitis (34). It has long been known that plasma C3 and C4 levels are significantly lower in patients with severe CHB than in healthy individuals or those with mild CHB (14). However, an in-depth mechanistic study of the involvement of the complement system in HBV-induced hepatitis is lacking. CFH is produced mainly in the liver. CFH competes with B or Bb for

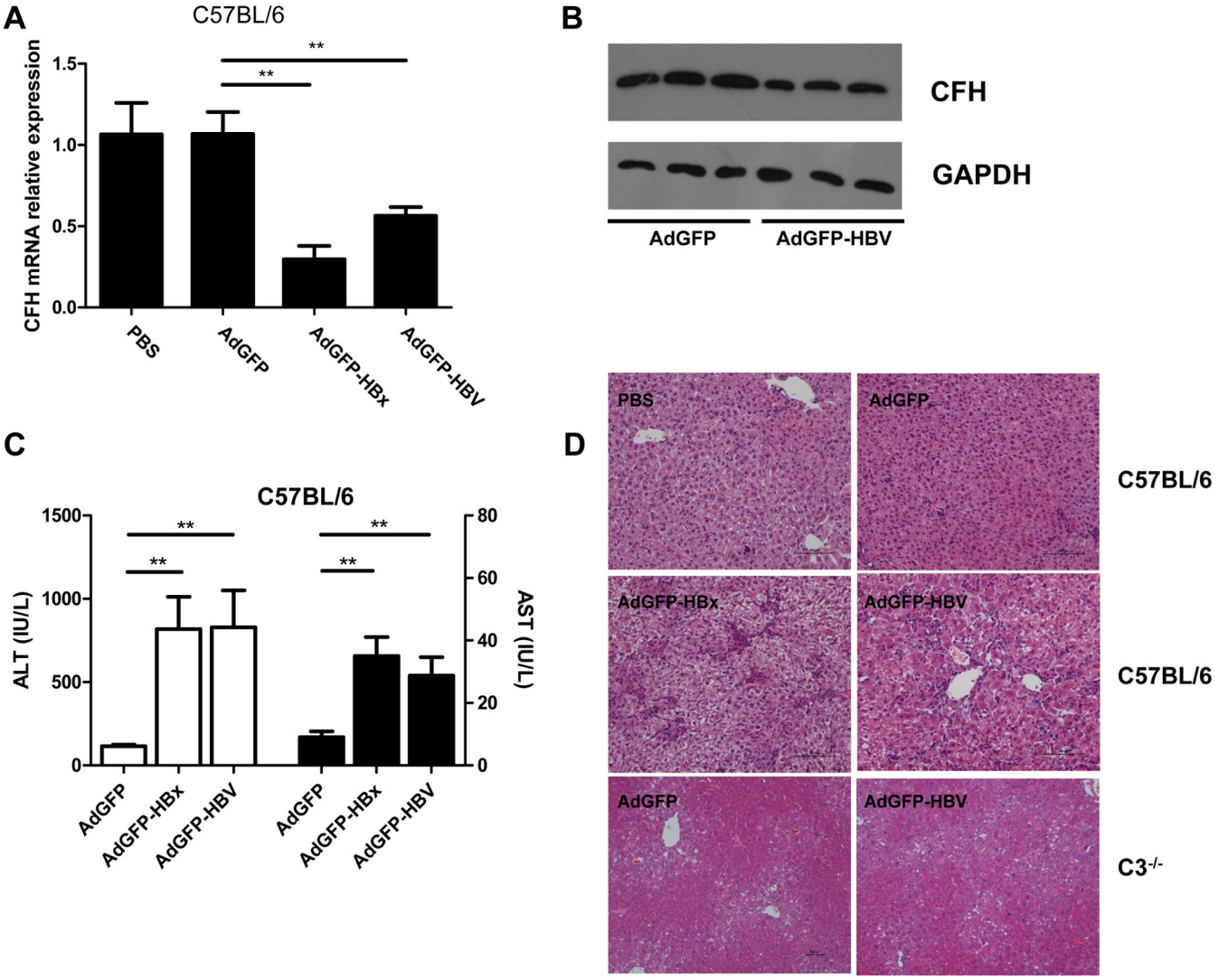

FIG 6 Reduced *CFH* expression is associated with HBV/HBx-induced liver inflammation. (A) Quantitative RT-PCR of the expression levels of *CFH* mRNA in C57BL/6 mice infected with AdGFP-HBx, AdGFP-HBV, AdGFP, or phosphate-buffered saline (PBS) via hydrodynamic injection; (B) immunoblot analyses of CFH protein in C57BL/6 mice infected with AdGFP or AdGFP-HBV; (C) the serum levels of ALT and AST in C57BL/6 mice infected with AdGFP, AdGFP-HBx, or AdGFP-HBV; (D) hematoxylin- and eosin-stained sections of the livers of C57BL/6 and C3$^{-/-}$ mice infected with the indicated adenoviruses or PBS. The mice were euthanized 7 days after the injection. Data are presented as the means ± SEM from $n = 5$ replicates. **, $P < 0.01$. Scale bar, 100 μm.

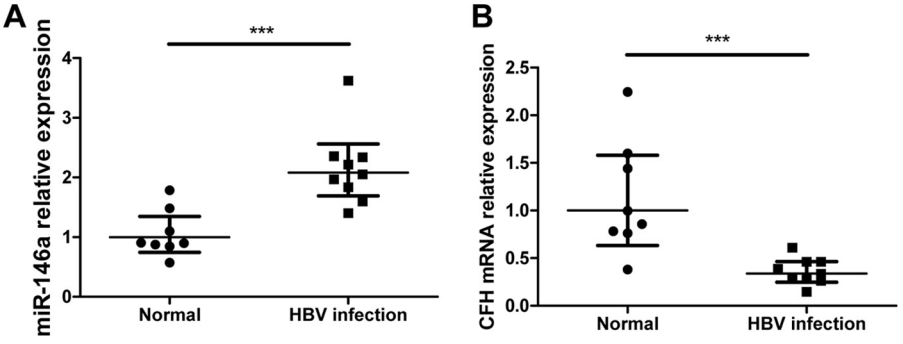

FIG 7 miR-146a and CFH are up- and downregulated, respectively, in HBV-positive cirrhotic human liver tissues. (A and B) The relative miR-146a (A) and *CFH* mRNA (B) expression levels in liver tissues from HBV-positive liver transplant patients with cirrhosis ($n = 9$) and healthy transplant donors ($n = 8$). Data are presented as the geometric mean with 95% CI. ***, $P < 0.001$.

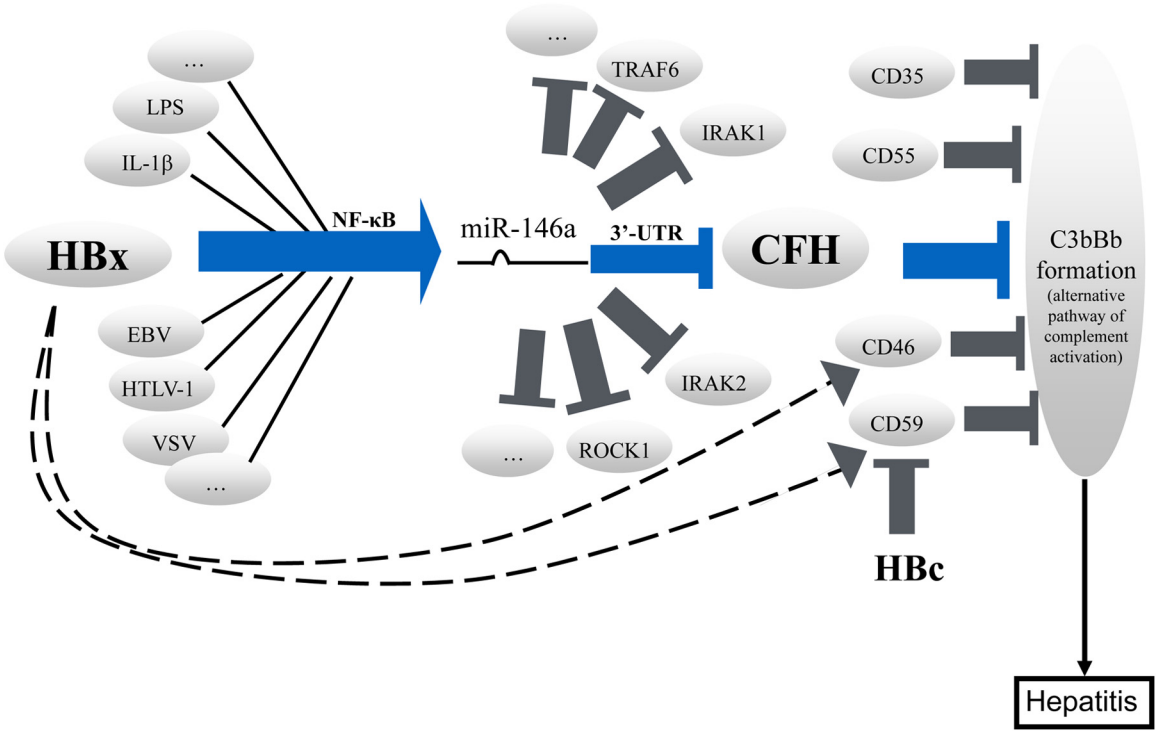

FIG 8 Overview of the regulation pathway from HBV infection to complement activation. HBx/HBV and several other viruses, such as Epstein-Barr virus (EBV), human T-cell lymphotropic virus 1 (HTLV-1), and vesicular stomatitis virus (VSV), as well as immune activators such as lipopolysaccharide (LPS) and interleukin 1β (IL-1β), can induce miR-146a expression by activating NF-κB signaling. miR-146a suppresses the expression of *CFH*, as well as multiple other genes, by binding to the 3' UTRs of its targets. Together with several other negative regulators of complement activation, CFH inhibits the formation of C3 convertase (C3bBb), thus inhibiting constitutive complement activation via the alternative pathway. HBx may also enhance the expression of CD59 and CD46, while HBc may inhibit CD59 expression. The HBx–miR-146a–CFH–complement activation pathway could play a central role in chronic HBV infection-related liver inflammation.

binding to C3b and accelerates the displacement of Bb from C3b, thereby inhibiting the formation of C3 convertase (C3bBb) in the alternative pathway (16). Constitutive high expression of CFH has been detected in the eye, and a *CFH* polymorphism is strongly associated with age-related macular degeneration (35); furthermore, CFH protects the eyes through recruiting to the surface of apoptotic cells, where it neutralizes the proinflammatory properties of these cells and halts complement activation (36). These reports imply a strong association of reduced levels of CFH at local sites with increased inflammation. Here, HBV infection of mice resulted in significantly lower *CFH* levels in hepatocytes and significant liver inflammation. Considering its role as an inhibitor of the alternative pathway of complement activation, we consider the lower CFH levels in hepatocytes to be a direct cause of the liver inflammation. Hepatic *CFH* expression was also reduced by HBx-induced overexpression of miR-146a. In agreement with our explanation of the connection between lower CFH levels and increased inflammation, we also observed increased liver inflammation in mice overexpressing HBx via a recombinant adenovirus vector (Fig. 5A and 6C). In addition, CFH expression was significantly lower in the livers of HBV-related cirrhosis liver transplant patients than in those of normal donors. Taken together, these results showed that reduced CFH expression induced by HBx plays a pivotal role in HBV-induced hepatitis.

The alternative pathway of complement activation has several negative regulators, including CFH, CD46, CD59, CD55, and CD35. HBV up- or downregulates several complement regulatory molecules (see Fig. 8). HBc sensitizes hepatocytes to complement-dependent cytotoxicity by downregulating CD59 (37, 38). In contrast, HBx upregulates CD46 (39) and CD59 (40) in hepatocytes to protect against inflammation. Such complicated and delicate interactions indicate the importance of complement activation in HBV immunopathogenesis; in addition, for complement regulators that are regulated by HBV proteins in opposing directions, such as the respective up- and downregulation of CD59 by HBx and HBc, it must be considered that either one might involve different degrees of regulation or require different cofactors. The overall effect of these regulations in the context of HBV infection might be related to the stage of disease progression. The balance between complement activation and inhibition by HBV might tilt at a certain point of CHB progression to favor complement activation rather than inhibition, resulting in the commencement of tissue damage. Generating a more complete understanding of the positive and negative regulation of complement activation might provide tools or methods to control complement-mediated liver injuries. In terms of therapeutic intervention for HBV-related hepatitis, the results presented here suggest that inhibiting complement activation in CHB patients would help to control chronic liver injuries. This subject will be the focus of future studies.

In summary, this study demonstrates that HBx upregulates miR-146a in HBV-infected hepatocytes by promoting NF-κB binding to the miR-146a promoter. Subsequently, miR-146a

TABLE 1 Oligonucleotides used for qRT-PCR of viral genomic and subgenomic RNA

Gene product or description	Application	Type	Sequence (5′→3′)
U6	Reverse transcription	Stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGTACGACAAAAATATG
	Real-time PCR	Forward	CGCAAATTCGTGAAGCGTTC
	Real-time PCR	Universal reverse	GTGCAGGGTCCGAGGTATTC
miR-146a	Pri-miR-146a clone	Forward	AGATCTTCAAGCGATCCTCCCACCACAG
	Pri-miR-146a clone	Reverse	CTGCAGCATGTAAAGCACTTACAACAGTACCTG
	Reverse transcription	Stem-loop RT	GCCTGGTCCACACCACCTGAGCCCGCACGACCAGGCAACCCATG
	Real-time PCR	Forward	AACGGCGGTGAGAACTGAATT
	Real-time PCR	Reverse	Universal reverse primer as above
	Promoter (−1132 to +21)	Forward	GCAGCTAGCTTTCGGTCCATGAGCACGT
	Promoter (−1132 to +21)	Reverse	GCAAAGCTTAGCGGTCAAGCGTCTTGG
	PromoterS1 (approx −1132 to −482)	Forward	GCAGCTAGCTTTCGGTCCATGAGCACGT
	PromoterS1 (approx −1132 to −482)	Reverse	CTCGAGAAGTGCGCGCAGGCTGAGTTTC
	PromoterS2 (−481 to +21)	Forward	GGTACCGAAAAGCCAACAGGCTCATTG
	PromoterS2 (−481 to +21)	Reverse	GCAAAGCTTAGCGGTCAAGCGTCTTGG
Mutation in NF-KB binding sites	146aS2mut	Forward	ACAGCAAAAGCCCGAGCGACCTTCGGTACCGAAAAGCCAACAGGCTCATTG
	146aS2mut	Reverse	CTTTCTCCAAGACGCTTGACCGCTAAGCTTGTAAGCGGATGCCGGGAGCAGAC
	146aS2mut	Mutant1	AGCCGATAAAGCTCTCACCATTTCCCCGCGGGGCTG
	146aS2mut	Mutant2	GCGCCGAGGAGGGATCTAGAAACCACTTTCCAGAGAGGGTTAGCG
CFH	Real-time PCR for humans	Forward	AACAGATTGTCTCAGTTTACCTAGC
	Real-time PCR for humans	Reverse	ACCCGCCTTATACACATCCTTC
	CFH-3'UTR	Forward	GAATCAATCATAAAGTGCACACC
	CFH-3'UTR	Reverse	GTTTTCCAGGATTTAATATGGTGC
	CFH-3'UTRmut	Forward	ACAGCAAAAGCCCGAGCGACCTTCGAATTCGAATCAATCATAAAGTGCAC
	CFH-3'UTRmut	Reverse	CCATATTAAATCCTGGAAAACCTCGAGCGGCTAAGGCACGCGCCACTTTT
	CFH-3'UTRmut	Mutant	TTAGTATTAAACTCTTGACTAATTTCATTTTTAAG
	Real-time PCR for mice	Forward	CTCGCTGTGTTGGACTTCCTT
	Real-time PCR for mice	Reverse	GTATGTAACCTCTTCCCCATGTTG
	Promoter	Forward	GGTACCTCAGCATTTCAATTTGTTGATTTTTG
	Promoter	Reverse	AAGCTTGGATCTTTTAAGAGGACATTTACCAG
GAPDH	Real-time PCR for humans	Forward	TGGGTGTGAACCATGAGAAGTATG
	Real-time PCR for humans	Reverse	ACTGTGGTCATGAGTCCTTCCA
	Real-time PCR for mice	Forward	TGCACCACCAACTGCTTAGC
	Real-time PCR for mice	Reverse	GTCTTCTGGGTGGCAGTGATG

downregulates *CFH* expression via 3′-UTR targeting. *In vivo*, the overall effect of this process is the promotion of liver inflammation. The HBx–miR-146a–CFH–complement activation regulation pathway might be the central mechanism of CHB-related immunopathogenesis. These findings might have important implications for the immunopathogenesis of CHB and the development of effective interventions.

MATERIALS AND METHODS

Plasmids and recombinant adenoviruses. The expression vector containing a 1.2-fold HBV genome (pHBV1.2), the pcDNA3.1-based expression plasmids containing the HBV genes encoding HBc, large surface protein, viral polymerase, or HBx, and the recombinant adenoviruses expressing HBV (AdGFP-HBV) and HBx (AdGFP-HBx) were generated as described previously (8). The PGL3-based promoter reporter plasmids (Promega) were generated by inserting the full-length human miR-146a promoter (nucleotides −1132 to +21 relative to the transcription start site), the 5′ (S1) segment of the human miR-146a promoter (nucleotides −1132 to −482), the 3′ (S2) segment of the human miR-146a promoter (nucleotides −481 to +21) (27), or the *CFH* promoter (nucleotides −553 to +110) (41) amplified from HepG2 cells. Site-directed mutagenesis of the two NF-κB binding sites in S2 was performed using the Multipoints mutagenesis kit (TaKaRa Biotechnology) (17). The pMIR-CFH3′ UTR and pMIR-CFH3′ UTR-M reporter plasmids were constructed by inserting the 3′ UTR (nucleotides +3748 to +3936) of *CFH* or its miR-146a binding site mutant into the pMIR-luciferase vector (8). The internal control reporter vector pRL-UBI was constructed by replacing the TK promoter region of the pRL-TK vector (Promega) with the ubiquitin promoter. The miR-146a expression plasmid (pIRES-EGFP-146a [where EGFP is enhanced green fluorescent protein]) was constructed by inserting an amplified pri-miR-146a sequence into the pIRES2-EGFP vector (Clontech). The antisense miR-146a expression plasmid (pSD14-miR-146a) was constructed by inserting an antisense oligonucleotide of mature miR-146a into the pSD14 vector (GeneChem Shanghai). The primers used in this study were synthesized by Invitrogen (Beijing) and are listed in Table 1.

Cell culture and transfection. HepG2, HepG2.2.15, HepAD38, and Huh-7 cells were cultured and transfected with plasmids as described previously (8). For the experiments shown in Fig. 3C and D, 30 μM (Fig. 3C and D) or 60 μM (Fig. 3C) pyrrolidine dithiocarbamate was added to the cells 6 h after transfection.

Quantitative real-time PCR. Total RNA was extracted and reverse transcribed as described previously (8). A 2-μl aliquot of reverse transcription product was used as the template for qPCR reactions using

GoTaq qPCR master mix (Promega). U6 snRNA and GAPDH were used for normalization of miRNAs and mRNAs, respectively. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method (42).

Reporter assays. For the experiments shown in Fig. 2 and 3, Huh-7 cells were cotransfected with the pGL3-146P, pRL-UBI, and pHBV1.2 plasmids or a pcDNA3.1-based plasmid containing the specific HBV gene; for the experiments shown in Fig. 5, Huh-7 cells were cotransfected with pMIR-CFH3′ UTR, pRL-UBI, and pIRES-EGFP-146a, pSD14-miR-146a, or pcDNA3.1-HBx. Dual-luciferase assays were performed as described previously (8).

Animals and ethics statement. The methods used to generate AdGFP-HBx- and AdGFP-HBV-infected mice and extract liver tissues of p21-HBx gene knock-in transgenic mice (C57BL/6) and control mice have been described previously (8). $C3^{-/-}$ mice (B6.129S4-$C3^{tm1Crr}$/J) have also been described previously (43). All animal-related procedures were approved by the Institutional Animal Care and Use Committee of the Beijing Institute of Microbiology and Epidemiology (permit no. BIME 2013-16), and relevant guidelines were strictly followed. Clinical liver tissue RNA samples were obtained from Dexi Chen of Beijing You'an Hospital. The study protocol was approved by the ethics committees of Beijing You'an Hospital.

Immunoblotting. Standard protocols were followed utilizing 8% denaturing gels, antibodies against human CFH (Genematix) and GAPDH (Cell Signaling Technology), a horseradish peroxidase (HRP)-conjugated secondary antibody, and ECL Western blotting substrate (Pierce).

Biochemical and histological analyses to evaluate liver injury. The test of levels of alanine aminotransferase (ALT) and aspartate amino transferase (AST) in mouse serum and histological analysis (hematoxylin and eosin staining) of mouse liver tissues were performed as described previously (43), and the extent of the liver damage was assessed by two independent observers who were blinded to the groups.

Statistical analysis. Each experiment was repeated at least three times. Comparisons of relative luciferase activity, as well as expression levels of miRNAs, mRNAs, and proteins between the two groups, were performed using Student's *t* tests (Fig. 1 to Fig. 6) or a Mann-Whitney test (Fig. 7). Quantitative values are, respectively, expressed as the means ± standard errors of the means (SEM) or geometric means with 95% confidence intervals (CI). *P* values of <0.05 were considered statistically significant.

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