

Original Article

The host gene *CSTF2* regulates HBV replication via HBV PRE-induced nuclear export

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Abstract

The persistent global burden of hepatitis B virus (HBV) infection has prompted ongoing investigations into host determinants of viral control. In this study, we investigate the regulatory influence of the host gene cleavage stimulation factor subunit 2 (CSTF2) on HBV replication dynamics. We demonstrate differential CSTF2 expression across the spectrum of HBV infection phases, with upregulated expression noted during the immune-reactive and inactive carrier states compared with the immune-tolerant phase. Notably, dose-responsive attenuation of HBV DNA, as well as surface and core protein levels, is observed subsequent to CSTF2 overexpression, whereas HBV RNA levels remain unaffected. Upon HBV transfection, a notable alteration in CSTF2 subcellular localization is discerned, suggesting active relocalization to the cytoplasm, potentially mediated through interaction with the HBV posttranscriptional regulatory element (PRE). This interaction appears to impede the nuclear export of HBV RNA. Additionally, distinct antiviral efficacies are attributed to the functional domains of the CSTF2 protein, indicating a multifaceted host defense mechanism. These insights increase the understanding of host-virus interplay and identify CSTF2 as a candidate for antiviral therapeutic strategies.

Key words hepatitis B virus, CSTF2, HBV PRE, immune tolerance, immune reactivity

Introduction

Hepatitis B virus (HBV) infection is a substantial public health challenge that contributes to considerable morbidity and mortality worldwide [1]. The natural history of chronic HBV infection is categorized into four distinct phases: immune-tolerant (IT), immune-reactive (IR), inactive carrier (IC), and HBeAg-negative active chronic hepatitis (ENH). Despite the apparent immunological quiescence shared by the IT and IC phases in chronic hepatitis B (CHB), these phases are characterized by divergent virological profiles. In the IT phase, individuals typically exhibit elevated viral loads exceeding 10⁷ IU/mL, whereas the IC phase is associated with markedly lower viral loads, often below 2000 IU/mL. These observations implicate the role of immune-independent mechanisms

in viral containment during phases of immunological dormancy.

In a previous investigation, we reported a significant elevation in intrahepatic mRNA level of the host gene cleavage stimulation factor subunit 2 (*CSTF2*) during the IC phase relative to the IT phase [2]. An inverse correlation was observed between CSTF2 expression and HBV replication, where CSTF2 downregulation was conducive to increased viral proliferation. CSTF2 forms an integral part of the cleavage stimulation factor (CSTF) complex, harboring an RNA recognition motif (RRM) essential for the precise processing of pre-mRNA [3]. The role of CSTF2 extends beyond that of HBV, with antecedent studies implicating its involvement in the modulation of human cytomegalovirus (HCMV) [4,5], human papillomavirus

(HPV) [6,7], and enterovirus 71 (EV71) [8,9] infections, underscoring its broader impact on viral pathogenesis.

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, is a hepatotropic and noncytopathic virus. Its structure is composed of an enveloped icosahedral nucleocapsid encapsulating a 3.2 kb partially double-stranded relaxed circular (RC) DNA genome. Previous research has illuminated the interaction between the polypyrimidine tract-binding protein (PTB) and the HBV posttranscriptional regulatory element (PRE), which is pivotal for the nucleocytoplasmic translocation of PRE-laden HBV mRNAs, thereby significantly influencing the operation and functionality of the HBV PRE [10]. In the context of HCMV infection, immunofluorescence assays have revealed considerable colocalization of the CSTF2 and PTB proteins. Along the infection continuum, the presence of PTB diminishes within viral replication compartments, accompanied by an increase in CSTF2 expression and a discernible reduction in PTB, which transitions from the nucleus to the cytoplasm [4].

Primarily localized within the nucleus, *CSTF2* encodes an RNA-binding protein that plays a pivotal role in the cleavage and polyadenylation of the 3' end of pre-mRNA [3,11,12]. Despite its well-established function in RNA processing, the relationship between CSTF2 and HBV infection has hitherto remained unexplored. Previous research has shown that the CSTF2 protein engages in a functional interaction with the HIV Rev protein and is upregulated by the Rev response element (RRE) [13]. The HIV Rev protein, a pivotal regulator in the late phases of viral replication, is predominantly localized in the cell nucleus and plays the primary role of interacting with RRE [14,15]. This interaction facilitates the nuclear export of HIV mRNA molecules containing RRE structures, thereby enabling downstream translation and viral assembly [16]. Intriguingly, the HBV genome harbors a structural element analogous to RRE, termed the HBV-PRE [17]. In a seminal study conducted in 1993, Huang *et al.* [18] identified this novel cis-regulatory element within the HBV genome that serves to inhibit the splicing process while facilitating the nuclear export of HBV transcripts, mirroring the functional attributes of the HIV Rev-RRE interaction [18–21].

A distinguishing feature of this interaction is that, unlike the HIV-1 Rev protein, whose nuclear export function is contingent upon the nuclear export signal (NES), the transport mechanism of the HBV PRE operates independently of the NES receptor-exportin 1 pathway [22]. Subsequent investigations revealed that PTB can specifically bind to the HBV PRE and dynamically shuttle between the nucleus and cytoplasm, thereby modulating the nuclear export of HBV mRNA containing PRE structures [10,23]. This functional parallelism between PTB-PRE and Rev-RRE interactions underscores their role in facilitating the nuclear export of viral transcripts.

In the present study, we demonstrated that CSTF2, as a host factor limiting viral replication, can potentially suppress HBV replication by inhibiting HBV PRE activity and disrupting RNA nuclear-cytoplasmic transport.

Materials and Methods

Cell culture and transfection

The human hepatoma cell line Huh7 (National Collection of Authenticated Cell Cultures, Shanghai, China) was maintained in DMEM (Gibco, Waltham, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, USA), 10 mM

HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were seeded in 12-well plates at a density of 1.0×10^6 cells per well. Each well was transfected with a total of 3 µg of plasmid via Lipofectamine 3000 (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The human hepatoma cell line HepG2.2.15, harboring the integrated dimer of the HBV genome (GenBank accession number: U95551, kindly provided by Prof. Ralf Bartenschlager of University of Heidelberg, Germany), was cultured in the same medium supplemented with 400 µg/mL G418 (Gibco, Waltham, USA).

Plasmids and reagents

The HBV replication competent plasmid pHBV1.3 (genotype D, subtype ayw, GenBank accession number V01460.1), in which the transcription of viral pregenomic RNA (pgRNA) is governed by an authentic HBV core promoter, was constructed as described previously [24]. The plasmids CSTF2-FLAG and five CSTF2-GFP deletions were synthesized by GeneChem (Shanghai, China). An siRNA designed to silence *CSTF2* (siCSTF2; SI05030585) and a negative control siRNA (siNC; 1022076) were purchased from QIAGEN (Dusseldorf, Germany). The PRE-mutated plasmid, namely, ayw1.3-PRE-mut, was engineered through four mutations in the loop region of PRE, which substitute the conserved C1305, G1307, G1308 and C1310 with A, T, T and A, respectively.

Viral nucleic acid analysis

Intracellular HBV core DNA and total RNA were extracted as described previously [24]. For DNA analysis, HBV core DNA was separated via electrophoresis on a 1.5% agarose gel and transferred onto a Hybond-XL membrane (GE Healthcare, Chicago, USA). For RNA analysis, 10 µg of total cellular RNA was resolved on a 1.5% agarose gel containing 2.2 M formaldehyde and transferred onto a Hybond-N+ membrane (GE Healthcare). The membranes were probed with either an α - ^{32} P-UTP-labeled minus or plus strand-specific full-length HBV riboprobe and then exposed to a phosphor imager screen. Hybridization signals were measured with a computerized imaging system (ImageJ software).

Western blot analysis

The cells were seeded in 12-well plates, washed twice with PBS, and then lysed in 200 µL of red loading buffer (Cell Signaling Technology, Boston, USA). Ten microliters of the cell lysate was then resolved via 12% SDS-PAGE, and the proteins were transferred onto nitrocellulose filter membranes (GE Healthcare). The membranes were blocked with 5% skim milk and incubated with antibodies against CSTF2 (ab200837; Abcam, Cambridge, UK), PTBP1 (ab133734; Abcam), HBsAg (ab9193; Abcam), HBcAg (ab8639; Abcam) and β -actin (Cell Signaling Technology). The membranes were washed with 1× TBST (as appropriate) and incubated with secondary antibodies (315-035-048, 111-035-045; Jackson ImmunoResearch, Lancaster, USA). Immunoreactive bands were visualized and captured with an enhanced chemiluminescence system (RPN2106; GE Healthcare).

Cell fractionation

The cells were seeded in a collagen-coated 35-mm-diameter dish in antibiotic-free DMEM/F12. After 6 h, each well was transfected with a total of 4 µg of plasmid with Lipofectamine 2000 (Life Technologies, Carlsbad, USA). For protein analysis, the cytoplasmic and

nuclear fractions of Huh7 cells were separated via the Qproteome Cell Compartment Kit (QIAGEN, Dusseldorf, Germany) following the manufacturer's directions. The purities of the cytoplasmic and nuclear fractions were confirmed by measuring the levels of cytoplasmic- and nuclear-specific protein markers (Annexin I and Lamin A/C, respectively) via western blot analysis following the manufacturer's procedures.

Luciferase reporter assay

A Dual-Glo luciferase reporter assay system (E2940; Promega, Madison, USA) was used to detect both firefly and Renilla (as internal controls) luciferase activity. The firefly luciferase reporter plasmids containing the HBV promoters pSP1, pSP2, pCP, pXP and the HBV element pPRE were generated and used as previously described [25]. The Huh7 cells were transfected with the CSTF2 plasmid (300 ng), vector plasmid (300 ng) or promoter reporter plasmid (100 ng) plus vectors (100 ng) via Lipofectamine 2000. Three days after transfection, the cells were harvested and lysed. Luciferase activities in the cell lysates were assayed via a dual luciferase assay system (Promega, Madison, USA) and measured via TopCount NXT (Packard, Meriden, USA). Relative firefly luciferase activities were normalized to Renilla luciferase activities.

Protein and RNA coimmunoprecipitation

Huh7 cells were cotransfected with CSTF2-FLAG and the control vector or with HA-tagged wild-type or mutant ayw1.3-PRE-mut. To obtain easily detectable levels of HBV RNA and CSTF2, the ratio between the input HBV plasmid and the CSTF2 expression vector was optimized to 3:1, and the transfection was maintained for 3 days. Cells were harvested and lysed on ice using a cell lysis buffer containing 1% NP-40, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 8% sucrose, and 1 U/mL RNasin Plus RNase Inhibitor (Promega, Madison, USA). After centrifugation to remove the cell debris, the clarified cell lysates were incubated with anti-FLAG affinity gel (Sigma-Aldrich, St Louis, USA) at 4°C for 2 h with gentle rotation. The beads were spun down and resuspended in rinse buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 10 mM ZnCl₂, and 1 U/mL RNasin Plus RNase Inhibitor) six times at 4°C. The pelleted beads were subjected to RNA extraction with TRIzol and protein sample preparation with Laemmli buffer.

Immunoprecipitated CSTF2 protein and HBV RNA were analyzed by western blot analysis and quantitative real-time PCR (qPCR) assay, respectively. In mRNA quantification, total cellular RNA was used to generate cDNA via the PrimeScriptRT Reagent Kit (TaKaRa, Dalian, China), as previously described [26]. cDNA was quantified in a Roche LightCycler 480 II using PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Primers used were as follows: 3.5 kb RNA forward primer 5'-GCCTTAGAGTCTCCTGAGCA-3' and reverse primer 5'-GAGGGAGTTCTTCTCTAGG-3', GAPDH forward primer 5'-TCAAGATCATCAGCAATGCCT-3' and reverse primer 5'-GATGATGTCTGGAGAGCCC-3'.

For CSTF2 and HBV PRE co-immunoprecipitation, a Magna RIP® RNA-Binding Protein Immunoprecipitation Kit (17-700; Millipore, St Louis, USA) was used following the manufacturer's directions. Cell lysates were prepared with RIP Lysis Buffer supplemented with protease and RNase inhibitors and incubated on ice. Protein A/G magnetic beads were pre-coated with 5 µg of anti-FLAG antibody (F7425; Millipore) and incubated with lysates overnight at 4°C. After six times wash with RIP Wash Buffer, bound RNA and

proteins were extracted. RNA was purified using phenol:chloroform and ethanol precipitation for the subsequent qPCR, while proteins were analyzed via SDS-PAGE and western blot analysis.

Immunofluorescence analysis

Huh7 cells were seeded on cover slips and transfected with plasmids or siRNAs. Forty-eight hours after transfection, the cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 10 min. The nuclei were stained with DAPI. CSTF2-FITC were stained with goat anti-rabbit IgG H&L (FITC) (ab6717; Abcam), and CSTF2-GFP deletions were stained with rabbit polyclonal to GFP (ab290; Abcam). The colocalization of CSTF2 (green) with DAPI was determined via a confocal microscope (LSM 710; Carl Zeiss, Jena, Germany) with the objectives Plan-Apochromat 63×/1.40 oil Iris M27. Images were visualized via ZEN acquisition software (2012; Carl Zeiss) and analyzed via ImageJ software.

Histological analysis and immunohistochemical staining

Pieces of liver tissues from patients at the IT, IR and IC phases were fixed in 10% (v/v) neutralized formalin. Pathological examination of tissue sections was performed by a collaborating pathologist at our hospital. For immunohistochemistry (IHC), paraffin-embedded liver tissues were rehydrated, boiled in 1 mM EDTA for antigen retrieval, and stained with DAB substrate from Invitrogen. After incubation with the CSTF2 antibody (1:200; Abcam), the IHC sections were scanned via the Aperio Scanscope CS (Aperio, San Diego, USA) and images were acquired at various magnifications.

All human subjects were recruited upon providing informed written consent. The study was approved by the Institutional Ethics Committee for Human Studies at Hua-Shan Hospital of Fudan University.

Statistical analysis

The data are shown as the mean ± standard error. All the experiments were replicated three or more times. We performed one-way ANOVA, including multiple comparisons, via GraphPad Prism 5 (GraphPad Software, San Diego, USA) and specified $P < 0.05$ as the standard for statistical significance.

Results

Intrahepatic CSTF2 expression was significantly elevated in subjects in the IR and IC phases compared with those in the IT phase

To investigate host factors that restrict HBV replication, we analyzed liver tissue samples from patients representing different disease phases. Subsequent to liver biopsies from 83 treatment-naïve patients across four natural-history phases, RNA extraction and microarray analysis were conducted. An outline of the clinical and virological characteristics of the CHB patients is provided in [Supplementary Table S1](#). In our previous study, we identified a group of host genes, including *CSTF2*, that were upregulated in the IC phase compared with the IT phase. CSTF2 may play a role in regulating HBV replication [2]. As shown in [Figure 1A](#), CSTF2 expression was significantly higher in the IR and IC phases than in the IT phase (IR vs IT, $P = 0.0388$; IC vs IT, $P = 0.0124$). Next, we investigated CSTF2 distribution *in vivo* via immunoprecipitation assays. For patients in the IT phase, CSTF2 was scattered in both the cytoplasm and nucleus, with relatively low expression levels, as indicated by representative images. Nonetheless, during the IR

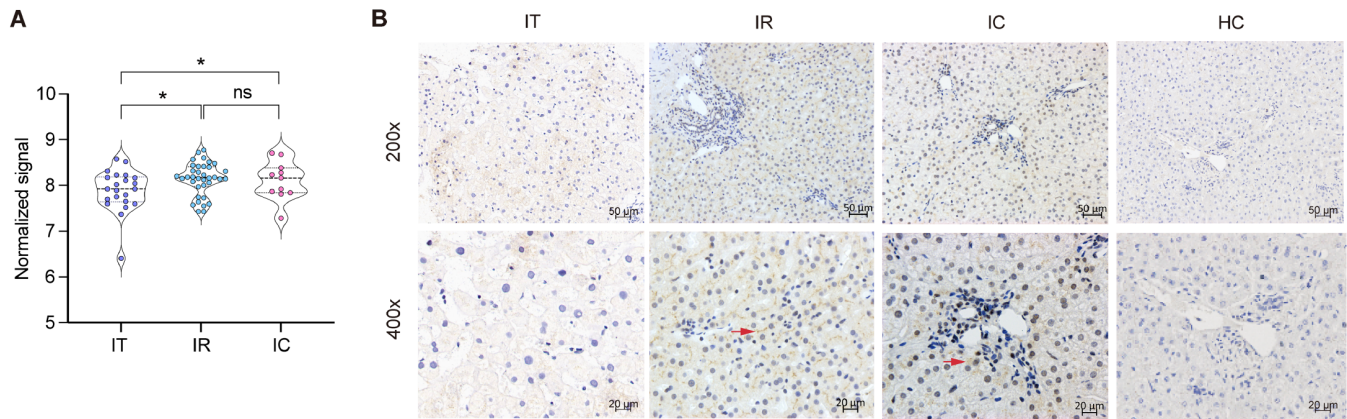


Figure 1. The intrahepatic expression of CSTF2 was significantly greater in patients in the immune-reactive and inactive carrier phases than in those in the immune-tolerant phase (A) Liver biopsies were obtained from patients and then subjected to RNA extraction and microarray analysis via the Affymetrix Human Genome U133 Plus 2.0 Array described in our previous studies [2]. The intrahepatic CSTF2 expression data are displayed in scatter plots. The mean expression levels of CSTF2 in immune-tolerant (IT), immune-reactive (IR), and inactive carrier (IC) patients were calculated and compared. A P value < 0.05 was considered significant. (B) Three sets of liver tissues from patients in the IT, IR and IC phases were fixed and stained with anti-CSTF2 antibody (yellow). * $P < 0.05$. ns, not significant.

phase, CSTF2 accumulated in the form of linear clustered coarse granules, implying the potential for the CSTF2 protein to aggregate and form a functional complex (Figure 1B). In the IC phase, the distribution of CSTF2 in the cytoplasm transformed into punctate clustered particles. The aggregation status of the CSTF2 protein suggests that CSTF2 may be activated by HBV during the IR phase and continue to exert antiviral effects in the IC phase.

Overexpression of CSTF2 inhibited HBV replication in cell cultures

To clarify the impact of CSTF2 on HBV replication, *in vitro* assays were conducted. Huh7 cells were subjected to transient transfection with a 1.3-mer construct of the HBV genome, along with CSTF2 siRNA, resulting in increased HBV DNA levels compared with those in Huh7 cells cotransfected with control siRNA (Figure 2A). Reduced expression of CSTF2 was confirmed by western blot analysis (Figure 2A, bottom panels), and no cytotoxic effects were observed. The silencing of CSTF2 led to modest increases in the levels of intracellular surface and core proteins (Figure 2A). Notably, the concentration of hepatitis B surface antigen (HBsAg) in the supernatant was significantly greater than that in the control groups (Figure 2B). While there was a marginal increase in HBeAg level, this increase did not reach statistical significance. Conversely, when a 1.3-mer construct of the HBV genome was cotransfected with either an empty vector or increasing concentrations of a CSTF2 expression construct, there was a dose-dependent decrease in the intracellular level of replicative HBV DNA (Figure 2C, top panels), as well as a reduction in the secretion of HBV proteins (Figure 2D, HBsAg, CSTF2 0.5 μg vs vector, $P = 0.0435$, CSTF2 1.0 μg vs vector, $P = 0.0359$, 1.5 μg vs vector, $P = 0.0048$; HBeAg, CSTF2 1.5 μg vs vector, $P = 0.0208$). Comparable trends were noted in terms of the intracellular levels of surface proteins and core proteins (Figure 2C, middle panels). Taken together, these results indicate that CSTF2 negatively regulates HBV DNA and proteins.

CSTF2 could regulate HBV replication via a posttranscriptional mechanism

To further elucidate whether CSTF2 regulates HBV replication

through transcriptional or posttranscriptional mechanisms, HBV RNA analysis and promoter reporter assays were performed. Knockdown of CSTF2 did not significantly alter the expression of HBV RNAs, including 3.5-kb pregenomic (pg) RNA, the precursor for HBV DNA replication, and 2.4/2.1 kb subgenomic RNAs for HBV envelope protein translation (Figure 3A). CSTF2 overexpression did not significantly decrease HBV RNA levels (Figure 3B). Moreover, CSTF2 did not markedly affect the activities of HBV promoters (Figure 3C). Therefore, we conclude that the inhibitory effect of CSTF2 on HBV replication is more likely to involve a posttranscriptional regulatory mechanism.

Changes in the intracellular distribution of CSTF2 in the presence of HBV

In this study, we observed distinct patterns of CSTF2 protein distribution in the liver tissues of CHB patients at different phases of their natural history. Therefore, we aimed to further investigate the changes in the subcellular CSTF2 protein distribution. In Huh7 cells, we employed immunofluorescence microscopy to observe the localization and distribution patterns of CSTF2. Our findings revealed that under non-HBV-transfected conditions, endogenously expressed CSTF2 was localized primarily within the nucleus (Figure 4A, top panels). However, in the context of HBV replication, endogenous CSTF2 exhibited a uniform distribution throughout the cytoplasm, indicating substantial alterations (Figure 4A, middle panels). Nevertheless, the majority of exogenously introduced CSTF2 (CSTF2-FLAG tagged plasmid) was expressed within the nucleus in the presence of HBV (Figure 4A, bottom panels). Upon isolation of cytoplasmic and nuclear proteins, we conducted western blot analysis. Exogenously transfected CSTF2 was distributed both within the nucleus and in the cytoplasm in the presence of HBV. Conversely, endogenously expressed CSTF2 exhibited a cytoplasmic distribution, with a minimal presence in the nucleus (Figure 4B). As predicted, in the absence of HBV replication, the level of endogenous CSTF2 protein in the nucleus was markedly lower than that during HBV replication. Exogenously induced CSTF2 was predominantly expressed in the cytoplasm (Supplementary Figure S1A). Thus, we conclude that in response to

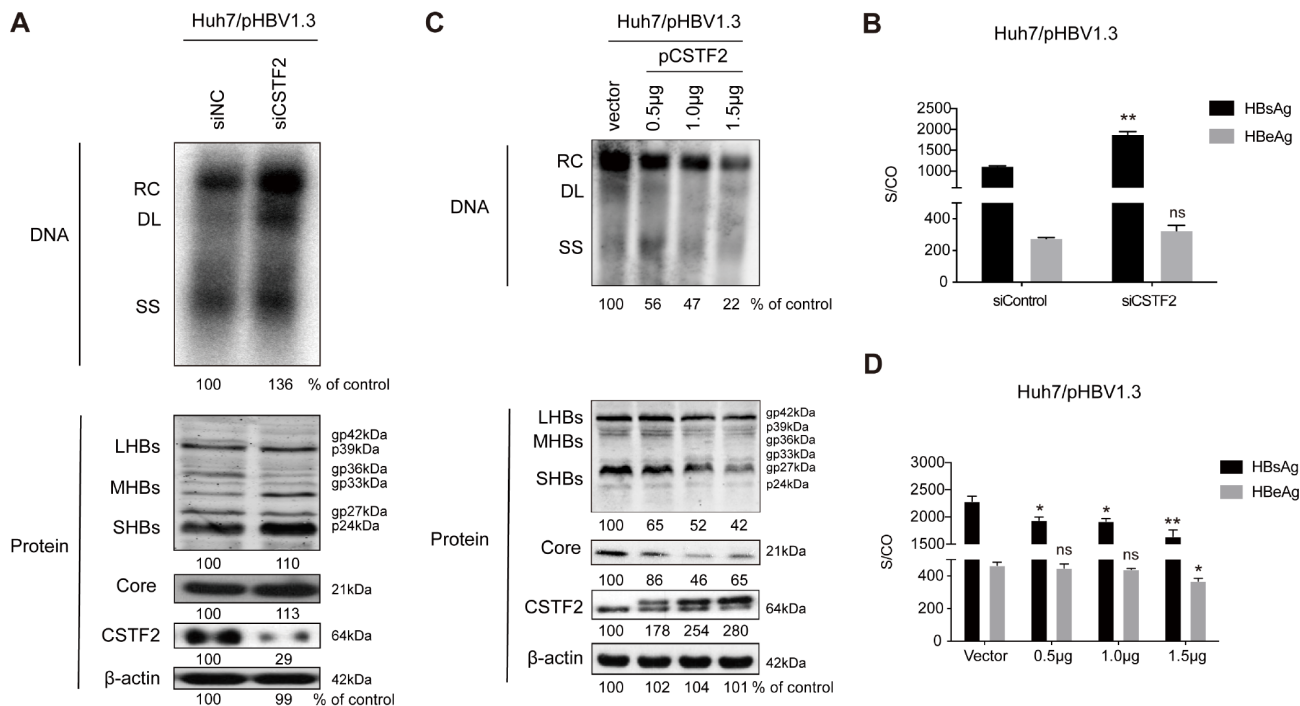


Figure 2. The impact of knocking down or overexpressing *CSTF2* on HBV replication in Huh7 cells (A) Huh7 cells in 12-well plates were cotransfected with 1.5 µg of pHBV1.3 containing 1.3-mer genome-length HBV sequences and 20 nM control siRNA or siCSTF2 and harvested after 72 h. HBV DNA and core proteins were analyzed via Southern blot (upper panels) and western blot analysis (lower panels), respectively. The positions of relaxed circular (RC) and single-stranded (SS) DNA are indicated. The expressions of intracellular viral proteins, including surface and core proteins as well as the CSTF2 protein, were also measured. The level of β-actin served as a loading control. The relative grayscale value for each lane of DNA and protein was measured via ImageJ software as the percentage of DNA in control cells. (B) Secreted HBsAg and HBeAg from the cell culture supernatant were measured and reported as S/CO. (C) Huh7 cells in 12-well plates were cotransfected with 1.5 µg of pHBV1.3 and the control empty vector or the constructed plasmid expressing flag-tagged pCSTF2 in a dose-dependent manner. HBV DNA and viral proteins, as well as the CSTF2 protein, were analyzed as described above. (D) Secreted viral proteins were detected as described above. **P* < 0.05, ***P* < 0.01. ns, not significant.

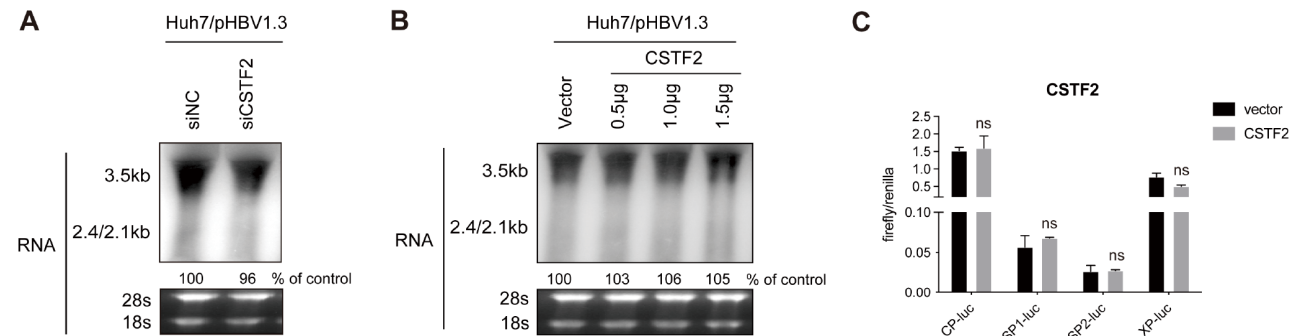


Figure 3. CSTF2 had no significant effect on HBV RNA or promoter activity (A,B) Huh7 cells in 12-well plates were cotransfected with 1.5 µg of pHBV1.3 and 20 nM control siRNA or siCSTF2 or with a control empty vector or a constructed plasmid expressing flag-tagged pCSTF2 in a dose-dependent manner. After 72 h, the cells were harvested, and HBV RNA was analyzed via Northern blot. Ribosomal RNAs (28S and 18S) are presented as loading controls. The positions of the 3.5 kb, 2.4 kb, and 2.1 kb HBV RNAs are indicated. The relative grayscale value for each lane of the RNA was measured via ImageJ software as the percentage of RNA in the control cells. (C) Huh7 cells in 24-well plates were cotransfected with 100 ng of four HBV promoters, Cp-Luc, SP1-Luc, or XP-Luc, and 100 ng of renilla plus 300 ng of control vector or pCSTF2. The cells were harvested 48 h posttransfection, and the luciferase activity was measured. The results are presented as the fold change compared with the vector group.

HBV stimulation, CSTF2 may engage in complex formation to exert antiviral effects.

CSTF2 inhibited HBV PRE activity and bound to 3.5 kb HBV RNA without affecting the protein expression level of PTB

Currently, certain host genes, such as *Mx1*, can inhibit the nuclear-

cytoplasmic transport of PRE-mediated preS/S RNAs [27]. Additionally, MyD88 is capable of degrading nuclear pgRNA and, through its interaction with PTB, reducing its protein expression level [28]. This disruption hinders the transport of preS/S RNAs, consequently affecting virus assembly and replication.

To support the HBV life cycle, we verified the influence of the PTB protein on HBV replication. Our results demonstrated that, upon

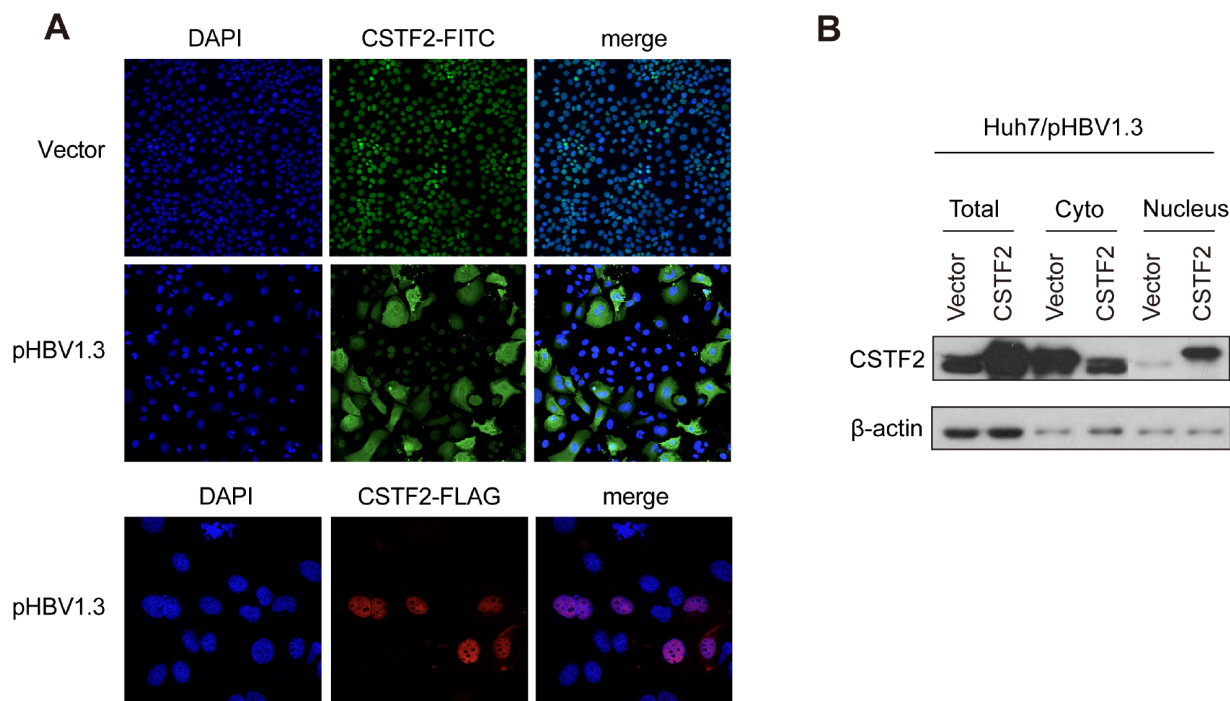


Figure 4. The intracellular distribution of the CSTF2 protein was altered in the presence of HBV (A) Huh7 cells in 12-well plates were transfected with 1.5 μ g of pHBV1.3 and control empty vector or constructed plasmids expressing flag-tagged pCSTF2. After 48 h, the cells were stained with FITC (green) and FLAG (red) antibodies and then fixed and stained with DAPI (blue). The distribution of exogenous and endogenous CSTF2 protein was observed via confocal microscopy. (B) Huh7 cells in 12-well plates were transfected with 1.5 μ g of pHBV1.3 and control empty vector or flag-tagged pCSTF2. The cells were harvested after 72 h. Cell fractionations for protein analysis were performed as described in “Materials and Methods”. The subcellular distribution of CSTF2 was revealed by Western blotting with anti-CSTF2 antibodies, with β -actin serving as a loading control (lower panels).

PTB knockdown, there was a reduction in HBV DNA levels, with no notable effect on 3.5 kb pgRNA or 2.4/2.1 kb preS/S RNA, which aligns with earlier findings (Supplementary Figure S2). To determine the influence of CSTF2 on the function of the HBV PRE, we generated HBV PRE plasmids incorporating a luciferase reporter gene alongside control plasmids. These constructs were subsequently cotransfected into the Huh7 cell line in conjunction with the CSTF2 overexpression plasmid and its corresponding control. Then, we evaluated the relative luciferase activity and computed the fold change. The results demonstrated that increased expression of CSTF2 considerably suppressed PRE activity (Figure 5A, $P = 0.0053$). In contrast, depletion of CSTF2 had no discernible effect on the activity of HBV PRE (Figure 5B). Therefore, we speculate that during HBV infection, CSTF2 may inhibit the nuclear-cytoplasmic transport function of PRE on HBV mRNAs, leading to a reduction in viral protein synthesis.

In the subsequent analysis, we explored the potential influence of CSTF2 on the expression level of the PTB protein. The results revealed that the presence or absence of HBV did not lead to any discernible effect on the expression of the PTB protein (Figure 5C). Furthermore, CSTF2 exhibited no discernible interaction with either the core protein or surface protein, as convincingly demonstrated through coimmunoprecipitation assays (Figure 5D,E).

The HBV PRE contains approximately 450 nucleotides encompassing nt 1151–1582 and is divided into three subelements: HPRE α (nt 1151–1346), HPRE β 1 (nt 1347–1457) and HPRE β 2 (nt 1458–1582) [29,30]. HBV RNA between 1241 and 1349 has two notable regions, the La protein binding site of nt 1275–1291 and another,

called the SL α stem loop, between nt 1292–1321 [30,31]. Notably, the CNGG tetraloop in the SL α structure was reported to be essential for SL α to interact with host proteins on the basis of the NMR resolution structure [30]. Thus, we speculated that HPRE α may play a role in the interaction with CSTF2. Four mutations in the loop region were made to replace the conserved C1305, G1307, G1308 and C1310 by A, T, T and A, respectively. When CSTF2 was cotransfected with the PRE-mutated plasmid (ayw1.3-PRE-mut), the inhibitory effect of CSTF2 on both the S and core proteins was greater than that of the wild-type pHBV1.3 plasmid (ayw1.3) (Figure 5F, HBsAg, $P = 0.0179$; HBeAg, $P = 0.0036$), which suggested that the inhibitory effect of CSTF2 on HBV proteins depends on the PRE element.

Surprisingly, CSTF2 exhibited greater binding to the 3.5 kb RNA containing the mutated PRE than to that containing the wild-type PRE, as shown in Supplementary Figure S1B ($P = 0.0069$). These findings indicated that CSTF2 may bind to 3.5 kb RNA but not directly to the PRE element. Thus, in light of the findings presented in Figures 2D and 5F, where the mutation of the PRE abolished the inhibitory effect of CSTF2 on HBeAg, it appears that CSTF2 has a specific inhibitory influence on precursor RNA. Furthermore, the full export capacity of PRE requires three subelements to work synergistically to transport HBsAg mRNA to the cytoplasm [30]. Consequently, CSTF2 may affect the translation of HBsAg and HBeAg by binding to 3.5 kb RNA and occupying PTB protein binding sites essential for the nuclear export of HBV RNA. Furthermore, CSTF2 inhibits PRE activity, affecting the nuclear export of preS/S RNAs and subsequently altering protein translation.

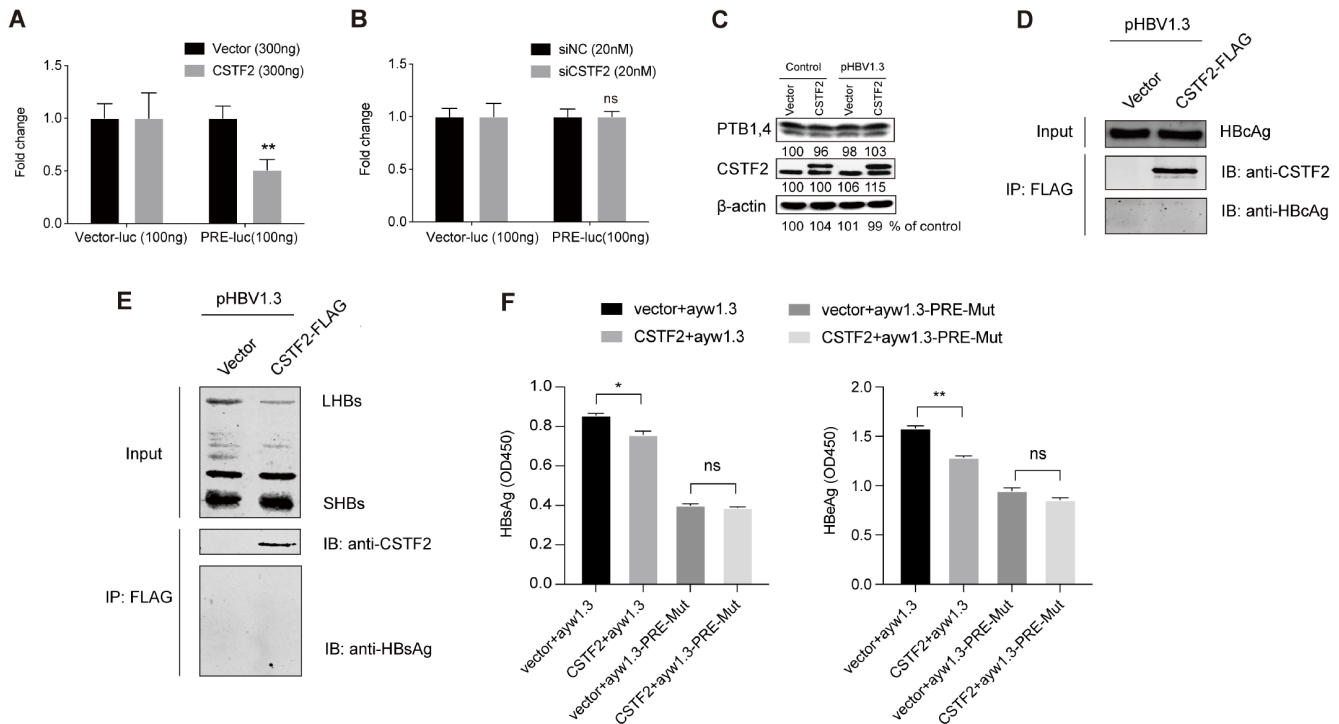


Figure 5. CSTF2 could directly inhibit HBV PRE activity without binding to viral proteins (A) Huh7 cells in 24-well plates were cotransfected with 100 ng of HBV PRE-Luc alongside control plasmids and 100 ng of renilla plus 300 ng of either the control vector or pCSTF2. The cells were harvested at 48 h posttransfection, and the luciferase activity was measured. The results are presented as the fold change compared with the vector group. (B) Huh7 cells in 24-well plates were cotransfected with 100 ng of HBV PRE-Luc alongside control plasmids and 100 ng of renilla plus 20 nM control siRNA or siCSTF2. The cells were harvested, and the data were analyzed as described above. (C) Huh7 cells in 12-well plates were transfected with 1.5 μ g of pHBV1.3 and control empty vector or flag-tagged pCSTF2. The cells were harvested after 72 h. Cell fractionations for protein analysis were performed as described in the Materials and Methods. The intracellular expressions of PTB and CSTF2 were assessed via western blot analysis with anti-CSTF2 and anti-PTB antibodies, with β -actin serving as a loading control (lower panels). (D,E) Huh7 cells in 12-well plates were transfected with 1.5 μ g of pHBV1.3 and control empty vector or flag-tagged pCSTF2. Coimmunoprecipitations were performed with beads covalently coated with anti-HBcAg or anti-HBsAg antibodies. Bonded CSTF2 proteins were analyzed by western blot analysis (as described previously). (F) Huh7 cells in 12-well plates were cotransfected with 1.5 μ g of Flag-tagged pCSTF2 and 1.5 μ g of the PRE mutated plasmid (ayw1.3-PRE-mut) as well as the corresponding vectors. Secreted HBsAg and HBeAg from the cell culture supernatant were measured and reported as the OD₄₅₀. * P <0.05, ** P <0.01. ns, not significant.

The functional domains of the CSTF2 protein play crucial roles in the inhibition of HBV replication

As previously reported, the CSTF2 protein is composed of an RNA recognition motif (RRM) responsible for RNA binding, a hinge domain that facilitates interactions with other members of the CSTF protein family, a proline/glycine-rich region, MEARA repeats, and a C-terminal domain (Figure 6A) [32]. The folding structure is shown in Figure 6B (<https://alphafold.ebi.ac.uk/entry/P33240>). Then, deletion plasmids containing these pertinent functional domains were constructed. Investigations conducted in Huh7 cells revealed that deletions encompassing the RRM domain resulted in notable decreases in HBV DNA levels. Intriguingly, deletions of amino acid sequences 100–577 and 379–577, which exclude the RRM domain, resulted in a varying degree of reduction in HBV DNA levels in comparison with other deletion mutants. This disparity suggests a contributory role of the MEARA domain in mediating HBV inhibition (Figure 6C). As a result, the levels of secreted HBsAg and HBeAg in the supernatants of cultured cells were significantly reduced by deletions encompassing the Hinge domain (Figure 6D). Compared with vector plasmids, deletions of aa 1–577 (P = 0.0078), aa 1–103 (P = 0.0093), aa 1–386 (P = 0.0017), and aa 379–577 (P = 0.0023) resulted in significant reductions. We subsequently

corroborated these findings in the HepG2.2.15 cell line. As expected, the deletion of amino acids 1–103 markedly decreased HBV DNA levels and significantly reduced the levels of secreted HBsAg and HBeAg (Supplementary Figure S3A,B). Next, we examined the distribution of these GFP-tagged deletions by immunofluorescence microscopy and observed that, in comparison with other deletions, deletions aa 1–103 and aa 379–577 were distributed in both the cytoplasm and nucleus (Figure 6E). As we have shown, the cytoplasmic distribution of CSTF2 suggested a potential antiviral effect against HBV. Notably, the RRM and MEARA domains may play crucial roles in inhibiting HBV DNA replication.

Overall, overexpression of CSTF2 resulted in a reduction in HBV DNA and protein levels, while the activity of the HBV promoter was unaffected. Notably, CSTF2 directly inhibited the activity of the HBV PRE element, independent of its interaction with the PTB protein, core protein, or surface proteins. We hypothesize that CSTF2 may competitively inhibit the functionality of the HBV PRE element, consequently hindering PTB protein-mediated nuclear–cytoplasmic transport of PRE-containing preS/S RNA, resulting in a reduction in surface protein synthesis. Concurrently, in the cytoplasm, CSTF2 appears to suppress the activity of PRE in its exported form, triggering the degradation of untranslated preS/S

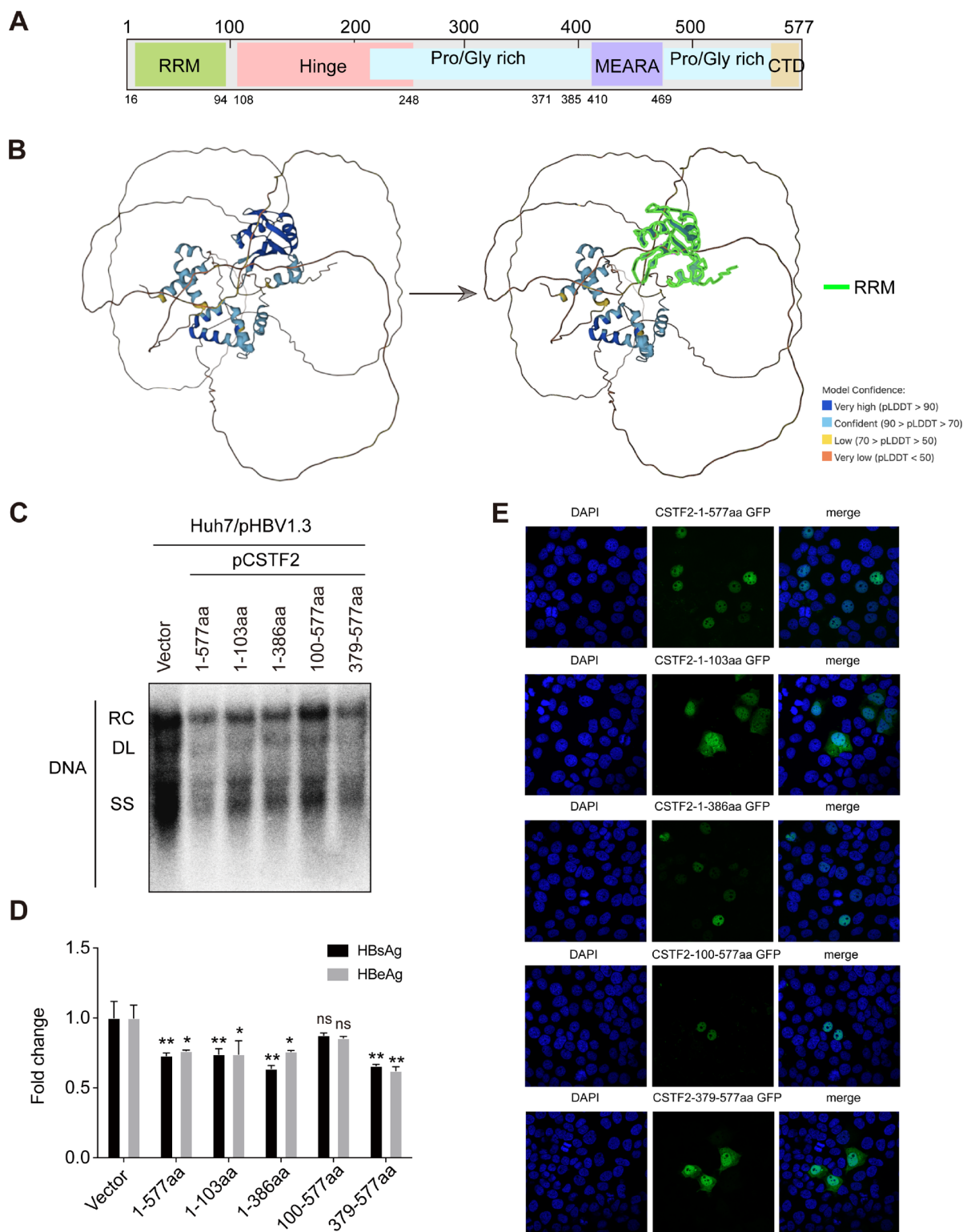


Figure 6. The functional domains of the CSTF2 protein play vital roles in the regulation of HBV replication (A) The primary structure of the CSTF2 protein. The protein encoded by *CSTF2* comprises 577 amino acids and mainly encompasses an RNA recognition motif (RRM), a hinge domain interacting with CSTF3, several domains rich in glycine/proline, the MEARA repeat and the C-terminal domain. (B) The secondary structure of the CSTF2 protein. (C) Huh7 cells in 12-well plates were transfected with 1.5 μ g of the pHBV1.3 or CSTF2 deletion plasmid or the control plasmid. The cells were harvested after 72 h, and HBV DNA was detected as described above. (D) Secreted HBsAg and HBeAg from the cell culture supernatant were measured and reported as fold changes. (E) Constructed CSTF2 deletion plasmids with GFP tags were cotransfected into Huh7 cells. After 48 h, the cells were stained with anti-GFP (green) antibodies and then fixed and stained with DAPI (blue). The distribution of CSTF2 deletion proteins was observed via confocal microscopy. * $P < 0.05$, ** $P < 0.01$. ns, not significant.

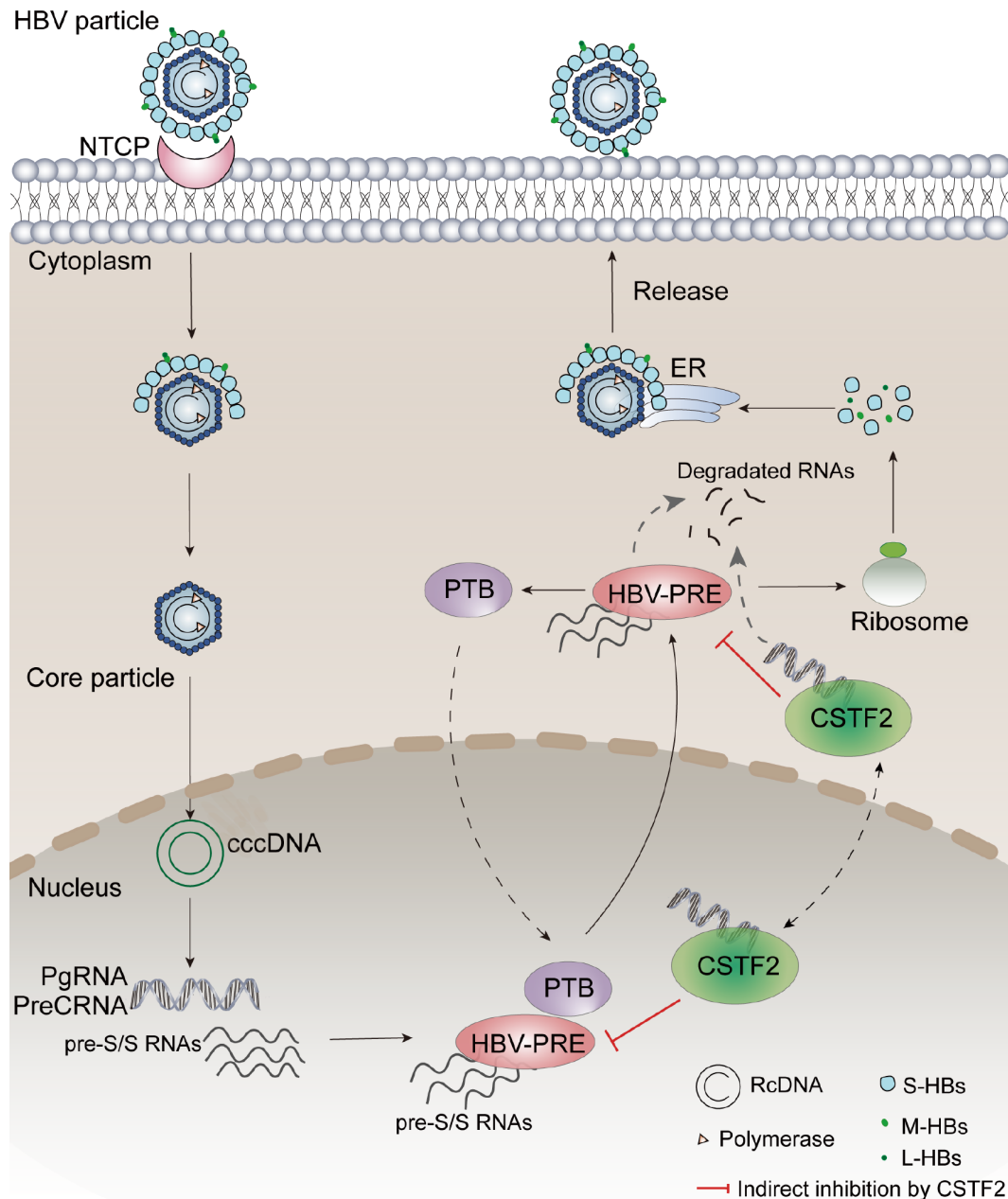


Figure 7. CSTF2 interferes with the life cycle of HBV CSTF2 has a direct suppressive effect on the activity of the HBV PRE element, which is devoid of interactions with the PTB protein, core protein, or surface proteins. It is hypothesized that CSTF2 might engage in competitive inhibition against the HBV PRE element, thereby obstructing the PTB protein-mediated translocation of PRE-containing preS/S RNA from the nucleus to the cytoplasm, which in turn diminishes surface protein synthesis. Simultaneously, within the cytoplasm, CSTF2 seems to curtail the activity of the exported form of PRE, instigating the degradation of untranslated preS/S RNA. Additionally, CSTF2 may affect the translation of HBcAg and HBeAg by binding to the 3.5 kb RNA and occupying PTB protein binding sites essential for the nuclear export of HBV RNA. This process subsequently influences the generation of Dane particles, culminating in the suppression of viral replication.

RNA in the cytoplasm. Additionally, CSTF2 may impact the translation of HBcAg and HBeAg by binding to 3.5 kb RNA, thereby occupying PTB protein binding sites crucial for the nuclear export of HBV RNA. This multifaceted process subsequently impacts the formation of Dane particles and ultimately leads to the inhibition of viral replication (Figure 7).

Discussion

Our present investigation revealed the compelling prospect that

CSTF2 serves as a critical regulator in modulating HBV replication. Functioning as a host restriction factor, overexpression of CSTF2 resulted in a marked decrease in both HBV DNA and protein levels, whereas its knockdown produced the opposite effects. Moreover, our findings strongly support the notion that CSTF2 attenuates HBV surface protein expression by directly targeting the PRE element, thereby providing a novel avenue for understanding host-virus interaction dynamics.

In recent years, several reports have shown that specific host

genes can modulate the nuclear export of HBV mRNA, thereby influencing the replication and assembly processes of the virus. Zinc finger CCCH domain-containing protein 18 (ZC3H18) has been shown to bind to the PRE and orchestrate the recruitment of TREX complex components for the nuclear export of the PRE via the NXF1-NXT1 pathway [33]. The TAR DNA binding protein (TARDBP) is posited to play a pivotal role in the export of unspliced pgRNA isoforms [34]. The HBV X protein engages in interactions with UAP56, which is potentially operative in the nuclear export of single-exon HBV mRNAs [35]. Similarly, MyD88 can degrade intranuclear pgRNA and attenuate PTB expression, thereby disrupting the transport of preS/S RNAs and consequently affecting viral assembly and replication [28]. In the present study, we elucidated that CSTF2 exerts a direct inhibitory effect on PRE activity, culminating in the impediment of the nuclear export of HBV RNA. Interestingly, the intracellular localization of CSTF2 significantly shifted following HBV infection. This observation suggests a reconfiguration of the nucleocytoplasmic transport pathway, potentially in response to viral manipulation of host cell processes. A more profound understanding of the nuclear export machinery employed by HBV mRNA could pave the way for the development of innovative targeted antiviral therapies indispensable for the management of hepatitis B. Our study demonstrated that CSTF2 appears to suppress the activity of the PRE element, triggering the degradation of untranslated preS/S RNA in the cytoplasm. Compared with the wild-type PRE, CSTF2 exhibited enhanced binding to the 3.5 kb RNA containing the mutated PRE instead of binding directly to the PRE element. We speculated that CSTF2 may impact the translation of HBcAg and HBeAg by binding to 3.5 kb RNA, thereby occupying PTB protein binding sites crucial for the nuclear export of HBV RNA. Currently, there are no studies on the mechanisms by which CSTF2 inhibits HBV replication; thus, we cannot compare our results with those of previous studies. We look forward to further investigations into the regulatory mechanisms of CSTF2 on HBV in the future.

Previous research has delineated the functional domains of CSTF2, highlighting the pivotal role of the RRM domain in RNA interactions. Our study builds upon these findings, demonstrating that the absence of the RRM domain leads to a diminished inhibitory effect on HBV replication. Additionally, the presence of MEARA repeats, followed by a proline/glycine-rich region and the C-terminal domain, was also found to exert antiviral effects. These effects were concomitant with alterations in the intracellular distribution of the protein, indicating a multifaceted role of CSTF2 in the host antiviral response. The MEARA repeats within CSTF2 consist of 12 motifs that adopt a helical conformation in solution, as evidenced by spectroscopic and calorimetric analyses. These helices, which are monomeric in nature and stabilized by electrostatic interactions, undergo an enthalpic change upon denaturation, quantified as 3.5 kJ/mol per residue. The precise mechanistic role of MEARA repeats in the inhibition of viral replication remains to be elucidated and warrants further study [36].

In summary, our study suggested that CSTF2 is a key host restriction factor that may modulate HBV replication through its inhibitory effect on PRE activity and probable binding to 3.5 kb HBV RNA. Although the exact mechanisms for the nuclear export of HBV mRNAs remain unclear, the novel function of CSTF2 is worthy of further investigation.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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