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# ADAR1 promotes HBV replication through its deaminase domain

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## TABLE OF CONTENTS



## 1. ABSTRACT

Adenosine deaminase acting on RNA1 (ADAR1) is an RNA editing enzyme that modulates the replication of several viruses. Here, we provide evidence showing that ADAR1 stimulates hepatitis B virus (HBV) DNA replication in hepatocellular carcinoma cell lines that are transiently or stably-transfected with HBV. We show that overexpression of ADAR1 promotes the replication of all four HBV genotypes (A, B, C, and D). Furthermore, analysis by mutagenesis shows that RNA editing region, and to a lesser content, RNA binding region of ADAR is responsible for the promotion of replication. Together, these data show that ADAR1 stimulates HBV replication.

## 2. INTRODUCTION

Hepatitis B virus (HBV) is a major cause of acute and chronic hepatitis worldwide. More than 350 million people suffer from chronic hepatitis B and annually 600,000 die of HBV-related disease and hepatocellular carcinoma (1, 2). HBV is noncytopathogenic, and infection of cells stimulates the host immune system. Additionally, certain

restriction factors within the host can confer resistance to HBV infection (3).

Adenosine deaminases acting on RNA (ADARs) converts adenosine (A) to inosine (I) by deamination of cellular and viral RNA substrates, and further modulates cellular biological process and viral replication (4, 5). As a RNA editing enzymes, ADAR1 has two isoforms, an interferon (IFN)-induced form (150-kDa, p150) and a constitutively expressed form (110-kDa, p110). P110 lacks the first 296 amino acids of the p150. Evidences show that both p110 and p150 are involved in the regulation of virus replication, and the regulation can be either proviral or antiviral, depending on the specific virus-host combination (4, 5). Moreover, the inconsistent effects of ADAR1 on Human Immunodeficiency Virus (HIV) (6-9) and Influenza Virus (IFV) were reported (10, 11). Intriguingly, ADAR1 could have a dual exclusive effect for Dengue Virus (DENV), with promotion at early time points post-infection and inhibition at later time points (12). The versatility of virus-specific role of ADAR1 deserves further exploration.

HBV is a 3.2 kb-long DNA virus that replicates via reverse transcription. Members of apolipoprotein B mRNA editing catalytic polypeptide (APOBEC) family inhibit HBV transcription and replication through DNA deaminase. Intriguingly, two members of the APOBEC family (A1 and AID) (13, 14) could also deaminate HBV RNA. However, as a typical RNA deaminase, roles of ADAR1 in HBV replication requires further analysis. Thus, experiment were carried out to address whether ADAR1 regulates HBV replication.

## 3. MATERIALS AND METHODS

### 3.1. Cell lines

Human hepatoma cell lines, Huh7 cells and HepG2 cells, the stable HBV-producing cell lines, HepG2.2.1.5 cells and HepAD38 cells were used. Huh7 and HepG2 cells were cultured in Eagle's minimum essential medium (MEM; HyClone, Utah, USA) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 1% L-glutamine, 1% Non essential Amino Acid; HepG2.2.1.5 and HepAD38 cells were cultured in Dulbecco's modified Eagle's medium (MEM; HyClone, Utah, USA) supplemented with 10% FBS, 1% Penicillin-Streptomycin. To turn off HBV replication, 1μg/ml tetracycline was added to the culture medium of HepAD38 cultures. All the cells were incubated in a 5% $CO_2$ incubator at 37ºC.

### 3.2. Plasmids, small interfering RNAs, and transfection

Plasmid PCH9/3091 is an HBV expression vector containing a CMV-early-promoter-driven 1.1 copies of an HBV genome (genotype D) (15). pCMV-HBV-gtA is an HBV genomic expression vector containing 1.2 copies of HBV (adw2) genome (Genbank X02763.1, genotype A) downstream of the CMV promoter in pBS (Promega)(33, 34). pCMV-HBV-gtB and pCMV-HBV-gtC are analogous HBV genomic expression constructs (From Dr. Shuping Tong, Shanghai Medical University, Shanghai, China) (16). The ADAR1 p110 and p150 constructs were generated with 3×HA tag and DDK tag, respectively. ADAR1 P150E/A and p150△R plasmids were used for the functional analysis as reported previously (17). P110-HA and p150-DDK point mutants were generated by PCR-based site-directed mutagenesis, and confirmed by sequencing. The mutations within the catalytic domain, resulting in amino acid change (H910A, E912A, C966A, and C1036A) (18) that abolishes the editing activity of ADAR1 protein. The mutant primers are listed in table 1. Specific small interfering RNA targeting ADAR1 mRNA (siRNAs) were purchased from Guangzhou RiboBio (GuangZhou, China) and transfected into cells using Lipofectamine 3000 (Invitrogen, Massachusetts, USA) according to manufacturer's instructions.

### 3.3. Western blot analysis

Fourth eight hours after transfection, cells were lysed in RIPA buffer (50mM Tris-HCl, pH7.4; 150 mM, NaCl; 1% NP-40) supplemented with a mixture of protease inhibitor cocktail (Roche, Berlin, Germany) and 1 mM PMSF for 30 min on ice. The lysates were clarified by centrifugation at 13,000×g for 5min at 4ºC. The clarified lysates were quantitated and denatured following the BCA manufacturer's protocol. The total lysates were separated on a 10%

**Table 1.** Primer sequences

| Gene name | Forward primer 5'-3' | Reverse primer 5'-3' |
|---|---|---|
| pgRNA | CCTAGTAGTCAGTTATGTCAAC | TCTATAAGCTGGAGGAGTGCGA |
| ADAR1 | GCCAAAGACACTCCCTC | GATTCCCTGTTCCCAAG |
| GAPDH | GAAGGTGAAGGTCGGAGTC | GAAGATGGTGATGGGATTTC |
| ADAR1-H910A | CAATGACTGCGCTGCAGAAATAATCTCCCGGAGAGG | CCTCTCCGGGAGATTATTTCTGCAGCGCAGTCATTG |
| ADAR1-E912A | TGCCATGCAGCAATAATCTCCCGGAGAGGC | GCCTCTCCGGGAGATTATTGCTGCATGGCA |
| ADAR1-C966A | TGTATATCAGCACTGCTCCGGCTGGAGATGGCG | CGCCATCTCCAGCCGGAGCAGTGCTGATATACA |
| ADAR1-C1036A | CGTACCATGTCCGCTAGTGACAAAATCCTACGCTGG | CCAGCGTAGGATTTTGTCACTAGCGGACATGGTACG |
| Abbreviation: pgRNA, pregenomic RNA | | |

SDS-PAGE and transferred to an Immobilon-P transfer PVDF membrane (Millipore). The membranes were blocked in 5% nonfat milk before incubating with specific antibodies. The antibodies used were anti-HA (1:500 dilution, Thermo, Ohio , USA), anti-DDK (1:500 dilution, OriGene, OriGene, Maryland, USA), anti-ADAR1 (1:1000 dilution, Abcam, Cambridge), anti-GAPDH (1:10000 dilution, ZSGB-Bio, Beijing, China). After incubation with the HRP-conjugated secondary antibody, the blots were visualized by ECL reagent (Solaibio Science and Tech Ltd., China). GAPDH was used as a housekeeping control.

### 3.4. Quantitative PCR analysis

Quantitative reverse transcription PCR (qRT-PCR) was performed to determine the mRNA levels. Total RNA was extracted with TRIzol reagent (Invitrogen, Massachusetts, USA) according to the manufacturer's instructions. 1μg total RNA was reverse transcribed into cDNA using PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan), followed by detection by qPCR in BIO-RAD CFX 96 (Bio-Rad, Washington,USA) system using the FastStart Universal SYBR Green Master (Roche, Berlin, Germany). The primers used are listed in Table 1. GAPDH was used as a housekeeping control

### 3.5. Southern blotting and qPCR detection for HBV total DNA

Intracellular viral core DNA of HBV was extracted 4 days after transfection as previously reported (15). Briefly, core-associated HBV DNA was collected by lysis of the cells, DNase I and proteinase K digestion, and phenol/chloroform extraction. The DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA). To detect HBV DNA by Southern blot analysis, the extracted viral DNAs were separated on a 1% agarose gel, transferred onto a nylon membrane (Roche, Berlin, Germany). The membranes were hybridized with a digoxigenin-labeled full-length HBV DNA probe using The DIG DNA Labeling and Detection Kit (Roche, Berlin, Germany) according to the manufacturer's instructions. The signal was detected by exposure on an X-ray film and scanning using the Versa Doc Imaging system (Bio-Rad, Washington,USA). Real-time PCR detection of HBV DNA was performed as previously described (16).

### 3.6. Statistical analysis

All the results were presented as mean±S.D. Student's *t*-test for paired samples was used for data analysis. Differences analyzed by GraphPad Prime 5.0 were considered statistically significant at a value of $P<0.05$.

## 4. RESULTS

### 4.1. ADAR1 knockdown down-regulated HBV replication

ADAR1 is endogenously expressed in human hepatoma cells. We first examined the effects of ADAR1 siRNA knockdown on HBV replication. As shown in Figure 1A, the expression level of HBV

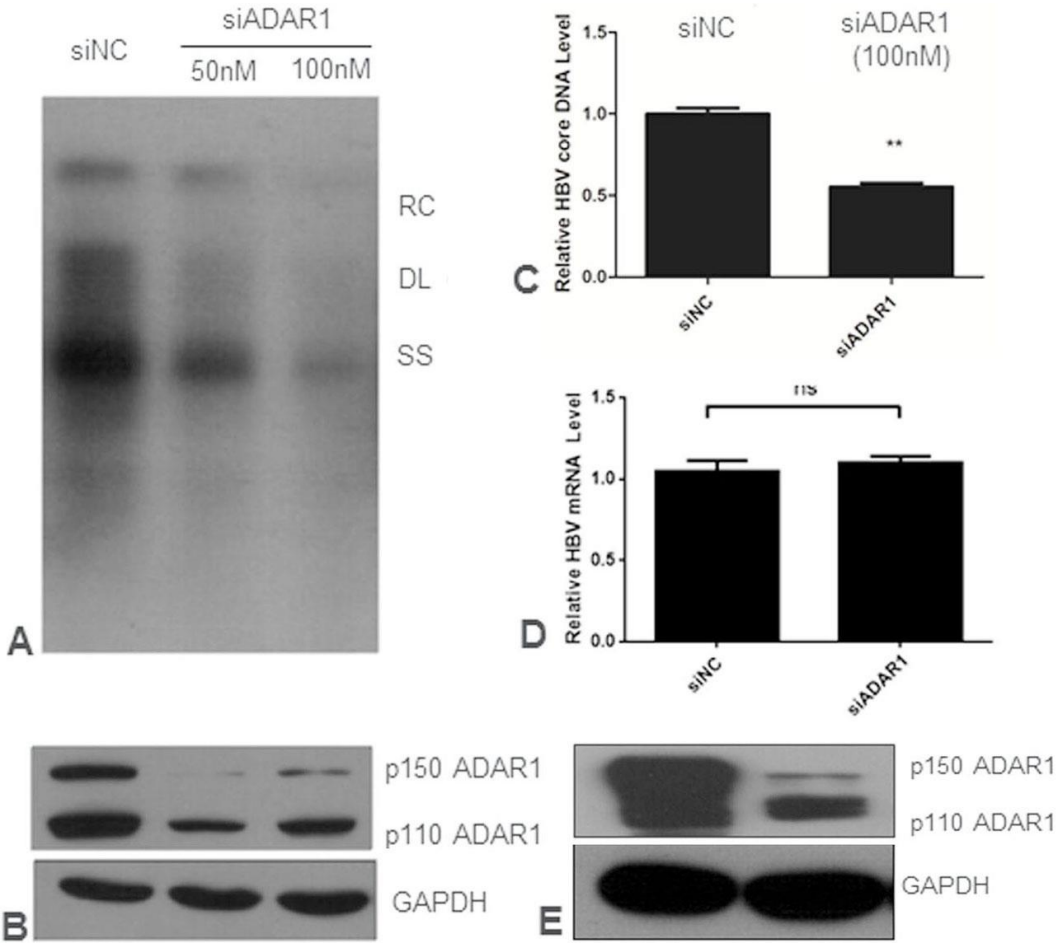


**Figure 1.** The knockdown of ADAR1 proteins decreased HBV replication. (A and B) Huh7 cells were transfected with 50 nM and 100 nM siADAR1 or siNC for 16h and then transfected with 2 ug of HBV plasmid pCH9/3091, and HBV replicative intermediates in the cells were evaluated at 48 h after transfection by Southern blotting (A) and the efficiency of ADAR1 siRNA knockdown was confirmed by Western blotting (B). (C, D, and E) Relative expression of HBV DNA (C) and RNA (D) in the HepG2.2.1.5 were detected by qPCR at 48h posttransfected with 100 nM ADAR1 siRNA. The levels of ADAR1 expression were detected by western blot (E). **P<0.01 from three independent experiments.

replicative intermediate (DNA) was substantially repressed with the ADAR1 knockdown. The repression was more obvious at 100 nM of ADAR1 siRNA than 50 nM (Figure 1A). Meanwhile, Levels of HBV DNA was reduced in HepG2.2.1.5 cells stably transfected by HBV by real-time PCR using primers targeting HBV core gene. The levels of HBV RNA showed no significant change (Figure 1B).

### 4.2. Over-expressed ADAR1 enhanced HBV replication

Further, an HBV expression plasmid was co-transfected with a control plasmid or ADAR1 (p110 and p150) in different human hepatoma cell lines, followed by HBV DNA and RNA detection. The level of HBV DNA replication increased significantly in both HepG2 cells and Huh7 cells (Figure 2A, B). Similarly, upon transfection of ADAR1 plasmid into two HBV stably replicative cell lines, HepG2.2.1.5 and HepAD38 cells, while HBV DNA was up-regulated, there was no significant change in the level of HBV RNA (Figure 2C.D).

### 4.3. ADAR1 increased the replication of HBV independent of genotypes

To examine the effect of genotypes on the promotion of ADAR1 on HBV replication, the three most common HBV genotypes (A, B and C)

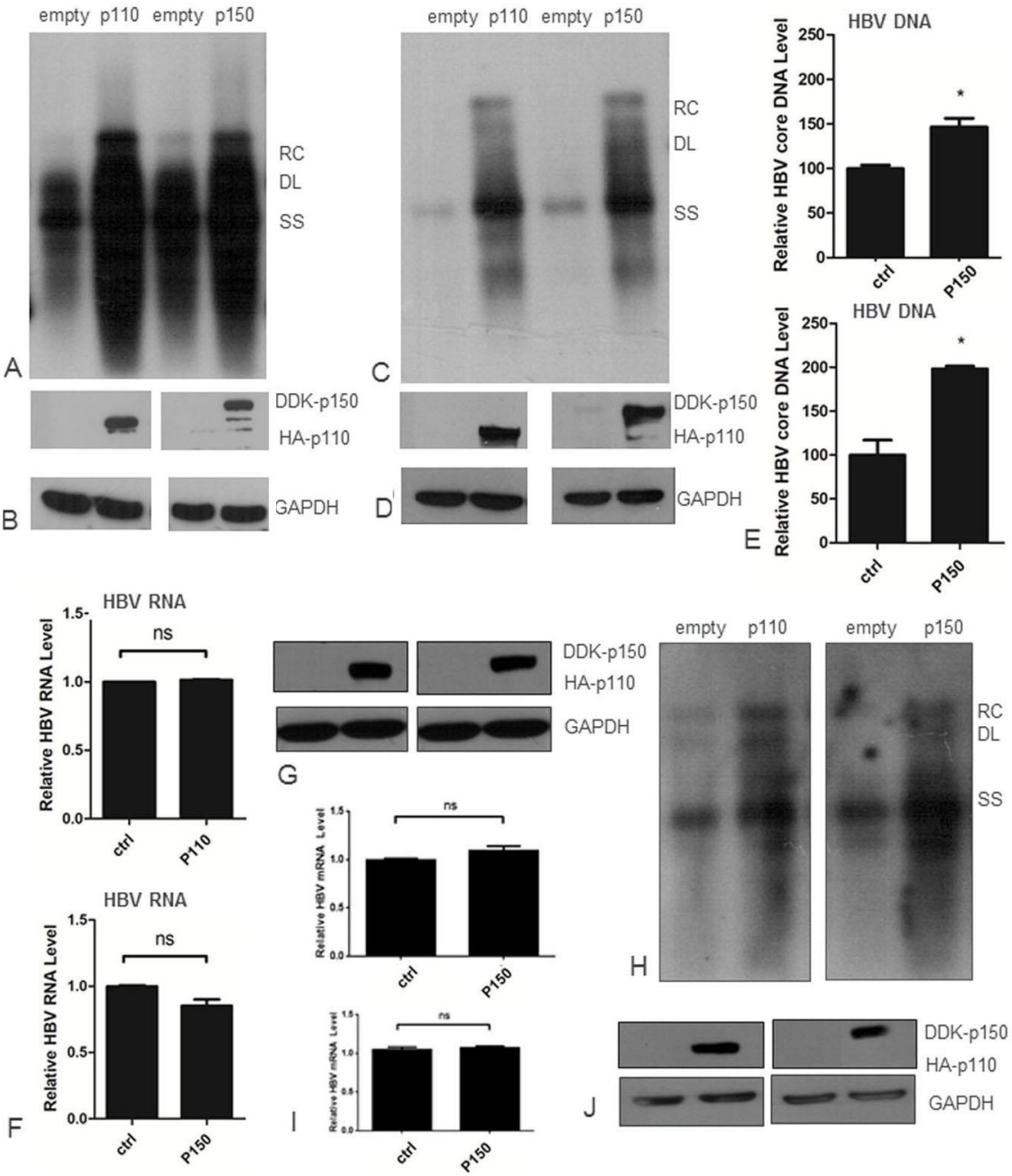


**Figure 2.** Overexpression of ADAR1 facilitates the replication of HBV. (A, B, C, and B) 0.4ug of HBV plasmid pCH9/3091 were cotransfected with 1.2ug empty and ADAR1 constructs (P110 or P150) into Huh7 cells (A and B) and HepG2 cells (C and D), HBV DNA replicative intermediates were detected by Southern blotting (A, C). (E, F, G, H, I, and J) 1.2ug ADAR1 constructs (P110 or P150) were transfected into HepG2.2.1.5 cells and HepAD38 cells, HBV DNA and mRNA were detected by qPCR in HepG2.2.1.5 cells (E and F) and southern blot in HepAD38 cells (H and I). The levels of ADAR1 protein were detected by western blot using anti-HA or anti-DDK antibody. GAPDH was used as an internal control. *P<0.05 from three independent experiments.

expression plasmids were co-transfected into Huh7 cells with ADAR1 plasmids, and then the HBV DNA were examined by Southern blotting. Results showed that both p110 and p150 isoform of ADAR1 promoted HBV DNA replication of all three genotypes that were examined (Figure 3A).

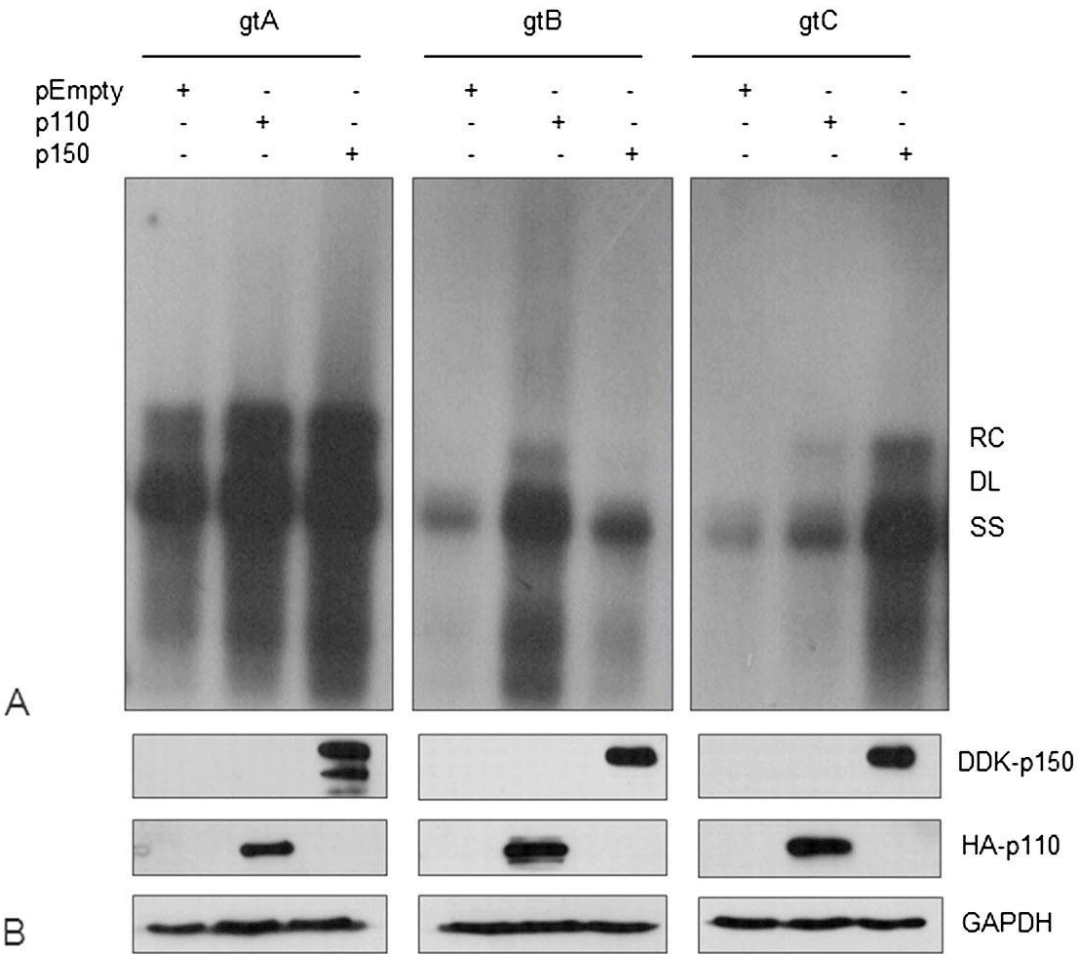


**Figure 3.** ADAR1 increased HBV replication of genotypes A, B and C. 0.4ug of genotype A, B or C HBV constructs were co-transfected with 1.2ug of P110 or P150 construct into Huh7 cells. HBV replicative intermediates were analyzed by Southern blot (A). ADAR1 expressions were confirmed by western blot using anti-HA or anti-DDK antibody (B). GAPDH was used as an internal control.

### 4.4. ADAR1 facilitated HBV replication through its editing function

Human ADAR1 has three different domains (Figure 4AB) that specify distinct functions of ADAR1 (4). We constructed domain mutants of ADAR1 and further explored the function of the mutated domain of ADAR1 on HBV replication in Huh7 cells (Figure 4A). Consistent with such results, as compared to empty vector control, ADAR1 p110, to a less degree, and the p150 isoform of ADAR1 enhanced HBV replication. Over-expressed ADAR1 p150 mutants (p150EA and p150ΔR) abolished the promoting effects of p150 isoform of ADAR1 on HBV replication (Figure 4C). This finding was consistent with the concept that RNA binding domain and RNA editing domain, solely or collectively, regulate ADAR1 function. It is reasonable to consider that RNA binding is a prerequisite for editing function.

To further determine the key amino acids responsible for the promoting effect of ADAR1 on HBV replication, we constructed four ADAR1 mutants harbored mutations of amino acids in the catalytic domain, and co-transfected them with HBV expressing plasmid into Huh7 cells (Figure 4A). As validated by southern blotting, both p110 and p150 isoforms of ADAR1, when over-expressed in Huh7 cells, increased HBV DNA replication (Figure 4C-D). However, over-expression of ADAR1 mutants (H910A, E912A, and C1036A) did not increase and rather decreased the HBV DNA replication (Figure 4D). C966A mutant showed a comparable or slightly

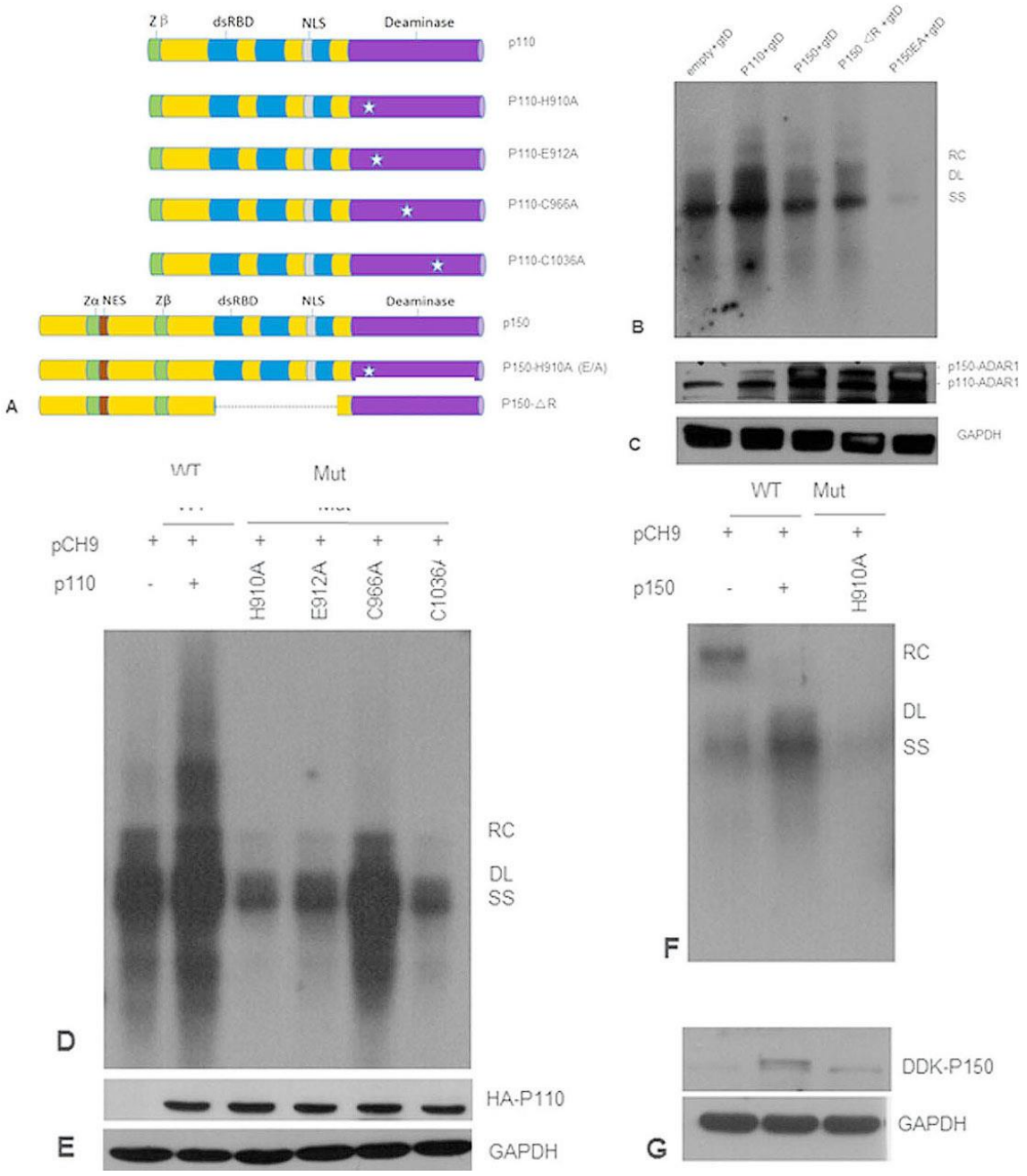


**Figure 4.** RNA editing and RNA binding domain of ADAR1 are required to promote HBV replication. (A) Schematic representation of human ADAR1 (p110 and p150) and its mutant constructs (p110H910A, p110E912A, p110C966A, p110C1036A, and p150H910A). The asterisks indicate the position of replaced amino acid in the catalytic domain of ADAR1. HBV replicative intermediates were detected by Southern blotting in Huh7 cells transfected with ADAR1 p110, ADAR1 p150 or its mutant expression plasmid (B, D, and F). Level of ADAR1 protein and its mutant constructs were confirmed by Western blotting using anti-HA or anti-DDK antibody (C, E, and G). GAPDH was used as an internal control.

increased level of HBV DNA replication as compared to the empty vector control. These data support the idea that HBV replication can be stimulated by ADAR1 through its editing activity, and conservative amino acids within the editing domain could play distinct roles during the replication.

## 5. DISCUSSION

ADAR1 is a multifunctional protein with editing-dependent and -independent activities, with the potential in regulating viral replication and innate immune response. In this study, we provide evidence that ADAR1 increases HBV DNA replication in human hepatoma cell lines. We further show that this enhancement relies on the deaminase activity of ADAR1. Both p110 and p150 isoforms of ADAR1 up-regulate HBV replication, as evidenced by ADAR1 siRNA knockdown and overexpression analysis in four widely-used cell lines (HepG2, Huh7, HepG2.2.1.5, and HepAD38), and further confirmed by the effect of ADAR1 on different genotypes. Previous work, by case-control studies in chronic HBV infection, indicated that the SNPs of ADAR1 gene correlate with the clearance of HBsAg (19). Our study provided solid and direct evidence that ADAR1 promotes HBV replication, adding the HBV to the list of viruses where their replication is supported by ADAR1. HBV and some other *Retroviridae* family viruses such as Equine Infectious Anemia Virus (EIAV) (20) and HIV (7) exhibit certain similarities in reverse transcription during replication. The fact that ADAR1 facilitates HBV replication in hepatocellular carcinoma cell lines that were transiently or stably-transfected with HBV suggests that it most probably acts as a downstream of pgRNA transcription. Recently, when incorporated into the HBV nucleocapsid, the deaminase such as A1 (21) and AID (14, 22), were found to deaminate HBV RNA. Additionally, the promotion of ADAR1 on HIV replication can be explained by the supportive role of virion assembly and gRNA packaging through the interaction of encapsidated ADAR1 and core proteins (p55 gag) (23-24).

Theoretically, ADAR1 ADAR1 might be encapsidated into the HBV nucleocapsid. Such encapsidation is in agreement with recent data showing that other cellular proteins, such as A1, AID, and DDX3, play a similar role (21-23). Furthermore, ADAR1 might stabilize the pgRNA by interfering with the function of the staufen1 protein, avoiding RNA decay in or out capsid, similar to the process during the cell apoptosis (25). ADAR1 might indirectly regulated HBV replication by modulation of cellular RNA processing and expression (such as encoding and noncoding RNA) (5, 12). Our findings demonstrate that the RNA editing domains, to a lesser extent, and the RNA binding regions, are the main functional regions responsible for the promotion of HBV replication. It is likely that ADAR1 RNA editing yields a quantity of inosine-containing RNA adequate to suppress innate immunity of host (5, 26-28), to modulate coding or noncoding RNA, and to affect the outcome of virus-host interaction (12, 29).

Highly conserved amino acids within the catalytic domain of ADAR1 are responsible for the deaminase activity (18, 30-31). Among these, $His^{910}$ and $Glu^{912}$ of ADAR1 are the key amino acids act respectively through zinc chelation and proton transfer (31-32). Substitution of $His^{910}$ with Gln/Gly ($H^{910}Y/Q$) and $Glu^{912}$ with Ala ($E^{912}A$) almost completely abolish A-to-I RNA editing activity or editing frequencies of verified A-to-I RNA editing sites through RNAseq (31-32). In addition, mutagenesis has revealed two conservative residues, $Cys^{966}$ ($C^{966}$) and $Cys^{1036}$ ($C^{1036}$), have been identified to play a role in zinc coordination along with $H^{910}$, and are necessary for ADAR1 deaminase activity (18). In our study, conversion of Cys to Ala at 966 ($C^{966}A$) results in a comparable or slight increase in the level of HBV DNA replication as compared to the empty vector control. $Cys^{966}$ likely has less effect on the catalytic activity of ADAR1 than other three residues ($His^{910}$, $Glu^{912}$, and $Cys^{1036}$). Although the precise roles of $Cys^{966}$ and $Cys^{1036}$ in HBV DNA replication is far from clear, they might play a role as zinc chelating ligands (18). In addition, while some conservative amino acid residues ($His^{910}$, $Glu^{912}$, and $Cys^{1036}$) are necessary for the enzyme function, $C^{966}$ provides tolerance to the amino acid conversion.

In this study, we demonstrate a supportive role of ADAR1 on HBV DNA replication in human hepatoma cell lines. We further prove that the enhancement relyies on the deaminase activity of ADAR1. Therefore, our findings point out a new host factor that is responsible for the enhancement of HBV replication.

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