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The Nucleotides on the Stem-Loop RNA Structure in the Junction Region of the Hepatitis E Virus Genome Are Critical for Virus Replication[▽]

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The roles of conserved nucleotides on the stem-loop (SL) structure in the intergenic region of the hepatitis E virus (HEV) genome in virus replication were determined by using Huh7 cells transfected with HEV SL mutant replicons containing reporter genes. One or two nucleotide mutations of the AGA motif on the loop significantly reduced HEV replication, and three or more nucleotide mutations on the loop abolished HEV replication. Mutations on the stem and of the subgenome start sequence also significantly inhibited HEV replication. The results indicated that both the sequence and the SL structure in the junction region play important roles in HEV replication.

Hepatitis E virus (HEV) is the causative agent of hepatitis E, and at least four major genotypes have been recognized in mammalian species: genotypes 1 and 2 are restricted to humans, whereas genotypes 3 and 4 are zoonotic (1–3, 9, 21, 26–29). The genome is a single-strand, positive-sense RNA molecule (12) consisting of a 5′ noncoding region (NCR), open reading frame 1 (ORF1) encoding the nonstructural proteins, ORF2 encoding the capsid protein, ORF3 encoding a small multifunctional protein (6, 22, 23, 31, 32, 38–40, 42), and a 3′ NCR. ORF2 and ORF3 are translated from a single bicistronic mRNA and overlap each other, but neither overlaps ORF1 (15, 19). The HEV genome contains two *cis*-reactive elements (CRE): the first CRE overlaps the 3′ end of ORF2 and the 3′ NCR and is essential for virus replication (13), and the second CRE may be the promoter for synthesis of the 2.0-kb subgenomic (SG) mRNA (14, 15). Graff et al. showed that neither ORF2 expression nor ORF3 expression was detectable when 6-nucleotide (nt) or 4-nt mutations were introduced into the junction region of the HEV genome; however, the roles of individual nucleotides in the junction region and its surrounding sequences in virus replication remain unknown (14).

We identified a region within the junction region (Fig. 1) of the HEV genome that shares nucleotide sequence identity with rubella virus and with the conserved alphavirus subgenomic promoter sequence. A highly conserved stem-loop (SL) structure was predicted to occur in the alphavirus junction region with sequences homologous to those of the HEV antigenome RNA in the junction region of the HEV genome (7, 19). The objective of this study was to determine the effect of mutations of the conserved nucleotides in the SL and its RNA structure on HEV replication.

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Analyses of the RNA SL structure and its surrounding sequences in the junction region of the HEV genome. RNA secondary structures often play important roles in viral replication, SG RNA synthesis, and translation efficiency (24, 36). It is believed that the complementary negative strand of the HEV SG promoter is recognized by RdRp or another viral component or host factor that interacts with RdRp, which then initiates the SG RNA synthesis in a primer-independent fashion at the SG start site (30). Using the mfold program (43), we identified two highly conserved SL structures in the intergenic region between the end of ORF1 and the start of ORF2 (Fig. 1C to E). The first SL is beyond the SG sequence (20) and thus may function as an SG promoter, and the SL also overlaps with the CRE region (14). Sequence analyses revealed an AGA triplet (in negative polarity) at nt −4 to −6 in the junction region (Fig. 1), positions similar to that of the AGA triplet in the rubella virus SG promoter (nt −8 to −10). The AGA triplet is also conserved in the SG promoters of alphavirus family members. Therefore, it is important to determine the function of the HEV AGA triplet and its surrounding nucleotides.

Both the sequence and structure of the SL in the HEV junction region are important for HEV replication. Although HEV infectious clones are available (13, 18, 35), the lack of an efficient cell culture for HEV prevents us from directly testing the replication of mutant viruses *in vitro* (34). It has been shown that a green fluorescence protein (GFP) HEV replicon is a good system to study HEV replication *in vitro* (10, 37). Therefore, in this study, we first constructed a genotype 1 HEV enhanced GFP (EGFP) replicon system (Y.-W. Huang and X. J. Meng, unpublished data) by using the Sar55 infectious clone (a gift from Sue Emerson, NIH, Bethesda, MD). We then constructed eight EGFP replicon-based HEV mutants and tested for their effects on HEV replication. Unfortunately, the sensitivity of the EGFP HEV replicon system was low (data not shown).

To definitively assess the roles of the nucleotides of the junction region in HEV replication, we subsequently con-

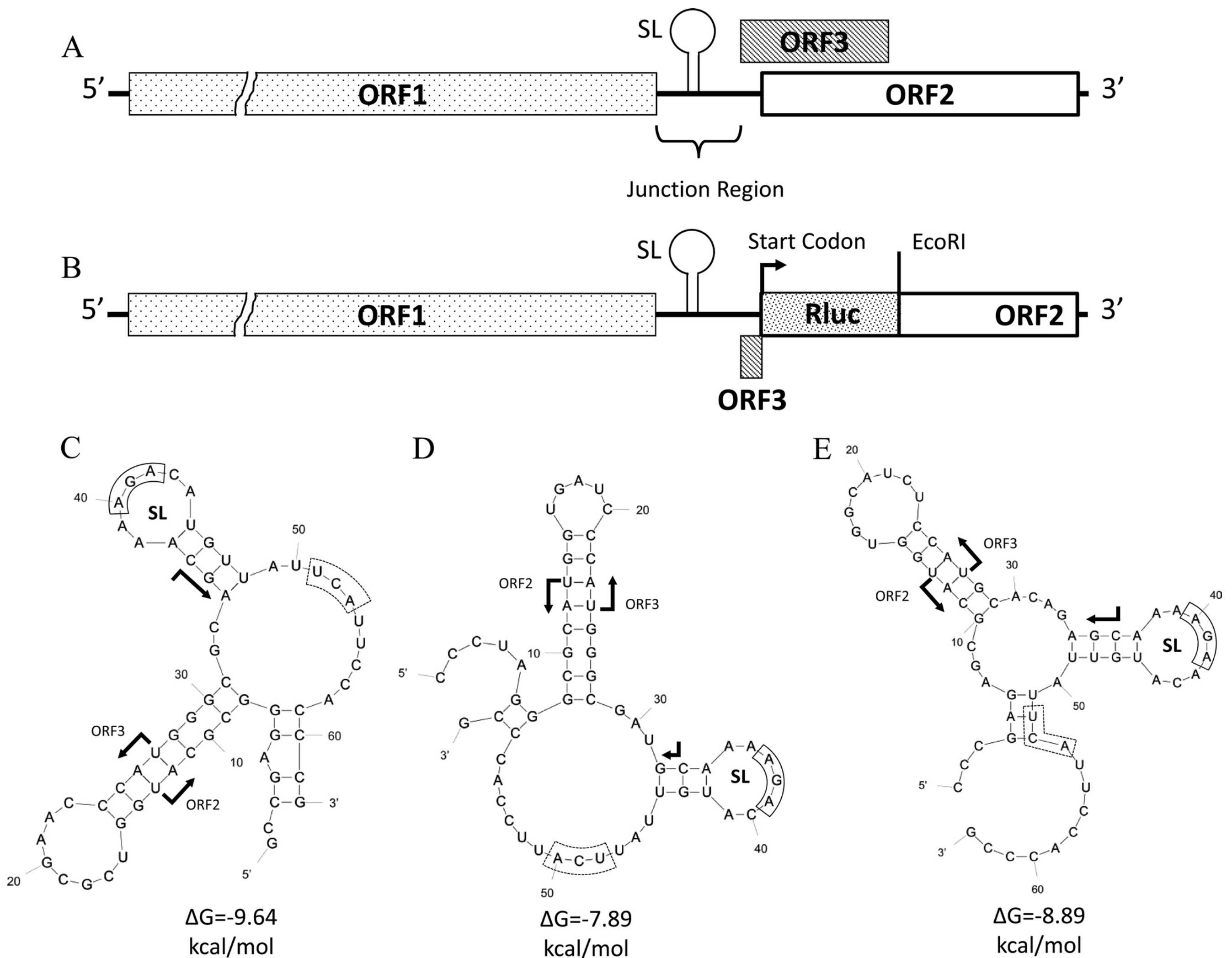

FIG. 1. (A) Organization of the HEV genome. The position of the predicted RNA stem-loop (SL) structure in the junction region is depicted. (B) Schematic diagram of the HEV Rluc replicon that was used as the backbone for the construction of various mutants. (C) Predicted secondary structure of the negative-polarity complement of the HEV genotype 1 (Sar55 strain) junction region. The sequence shown extends from nt 5096 through 5157. (D) Predicted secondary structure of the negative-polarity complement of the HEV genotype 3 (pSHEV-3 strain) junction region. The sequence shown extends from nt 5142 through 5200. (E) Predicted secondary structure of the negative-polarity complement of the HEV genotype 4 (T1 strain) junction region. The sequence shown extends from nt 5138 through 5200. The conserved AGA triplets in the SG promoters of alphavirus family members are boxed with solid lines. The HEV subgenome start site is indicated with arrows. The start sites of ORF2 and ORF3 are also indicated; the stop codon of ORF1 is boxed with dotted lines.

structed a novel HEV replicon system by replacing nt 5148 to 5816 of the infectious clone pSK-HEV-2 with the *Renilla* luciferase (Rluc) gene (Fig. 1B). By utilizing the start codon of HEV ORF2, the Rluc HEV replicon expresses *Renilla* luciferase (Rluc) (Fig. 1B), which was used as a reporter for quantifying HEV replication. By using the Rluc HEV replicon as the backbone, we constructed 11 SL mutants, designated as follows (Fig. 2A; Table 1) (sequences shown as the negative polarity complement of the HEV genome): M1 (A5118→U); M2 (C5122→U, G5123→U); M3 (AGA5116 to -5118→UCU); M4 (AAAGA5116 to -5120→UUUCU); M5 (UGUU5110 to -5113→ACAA), which contains mutations on one leg of the SL stem; M6 (AA5119 to -5120→UU); M7 (C5101→G); M8 (C A5124 to -5125→AU); M9 (AGCA5121 to -5124→UUGU), which contains mutations on another leg of the stem; M59 (UGUU5110 to -5113→ACAA, AGCA5121 to -5124→UUGU), with mutations on both legs of the stem; and M78 (C5101→G, CA5124 to -5125→AU), which is a combination of M7 and M8. In addition, the HEV Rluc replicon mutant with a GDD→GAA mutation on RdRp (MGAA) was constructed and used as a negative control. Capped RNA transcripts from each of the 11 mutant replicons along with the MGAA and wild-type replicon were synthesized *in vitro* with an mMessage mMachine T7 kit (Ambion) (17, 19, 37). The capped RNA transcripts of each mutant and control were transfected into the Huh7-S10-3 liver cell line (a gift from Sue Emerson, NIH, Bethesda, MD) (11, 15) with 1,2-dimyristyl Rosenthal inhibitor ether (DMRIE-C) reagent (Invitrogen). The luciferase activities were measured with a dual luciferase reporter assay system (Promega) at 5 days posttransfection. Firefly luciferase RNA

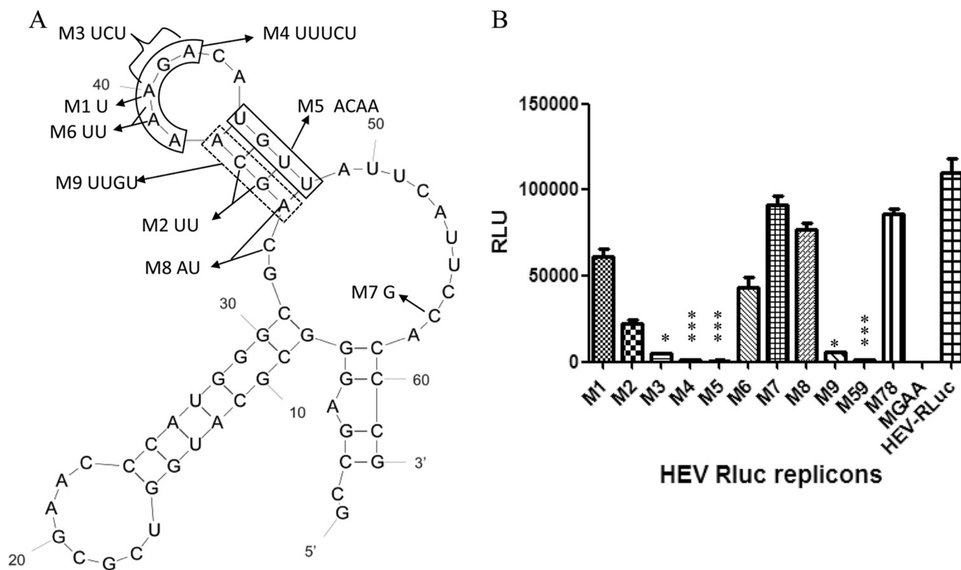

FIG. 2. Mutational analyses of the predicted stem-loop (SL) structure in the junction region of the HEV genome. (A) Mutations were introduced into the stems and loop sequences of the SL structure in mutants M1 to M9, M59, and M78 for the HEV Rluc replicon. (B) Relative luciferase activities in Huh7 S10-3 cells transfected with HEV Rluc mutants M1 to M9, M59 (i.e., M5 plus M9), M78 (i.e., M7 plus M8), HEV Rluc MGAA (negative control), and the wild-type Rluc replicon (HEV-RLuc). The relative luciferase activities are shown at 5 days posttransfection and normalized with cotransfected firefly luciferase RNA. Data are from an average of eight separate replicate experiments, and the error bars indicate standard deviations (SD). The differences in signal produced by HEV Rluc mutants and the wild-type Rluc replicon were compared by one-way analysis of variance (ANOVA) using the Kruskal-Wallis test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; RLU, relative light units.

was cotransfected with HEV Rluc replicon RNAs to normalize the *Renilla* luciferase signal.

The results showed that the Rluc signal is lower in cells transfected with RNA of mutant M1, M2, M3, M4, M5, M6, M9, or M59 than that in cells transfected with RNA of the wild-type HEV Rluc replicon (Fig. 2B), with statistically significant differences for mutants M3, M4, M5, M9, and M59. The mutant M3, which changed only the AGA motif, abolished

TABLE 1. Primers used in the generation of HEV mutants and LNAs used for inhibition of HEV replicon replication

Oligonucleotide	Polarity[a]	nt position[b]	Sequence[c]
Mutagenic primers			
HEV2m1R	−	5090–5139	5′-CGAACCCATGGGCGCAGCAAA**T**GACATGTTATTCATTCCACCCGACACAG-3′
HEV2m1F	+		5′-CTGTGTCGGGTGGAATGAATAACATGTC**A**TTTGCTGCGCCCATGGGTTCG-3′
HEV2m2R	−	5097–5141	5′-CGCGAACCCATGGGCGCA**TT**AAAAGACATGTTATTCATTCCACCC-3′
HEV2m2F	+		5′-GGGTGGAATGAATAACATGTCTTTT**AA**TGCGCCCATGGGTTCGCG-3′
HEV2m3R	−	5094–5140	5′-GCGAACCCATGGGCGCAGCAAA**TCT**CATGTTATTCATTCCACCCGAC-3′
HEV2m3F	+		5′-GTCGGGTGGAATGAATAACATG**AGA**TTTGCTGCGCCCATGGGTTCGC-3′
HEV2m4R	−	5094–5140	5′-GCGAACCCATGGGCGCAGCA**TTTCT**CATGTTATTCATTCCACCCGAC-3′
HEV2m4F	+		5′-GTCGGGTGGAATGAATAACATG**AGAAA**TGCTGCGCCCATGGGTTCGC-3′
HEV2m5R	−	5085–5134	5′–CCATGGGCGCAGCAAAAGACA**ACAA**ATTCATTCCACCCGACACAGAATTG-3′
HEV2m5F	+		5′-CAATTCTGTGTCGGGTGGAATGAAT**TTGT**TGTCTTTTGCTGCGCCCATGG-3′
HEV2m6R	−	5090–5139	5′-CGAACCCATGGGCGCAGCAA**TT**GACATGTTATTCATTCCACCCGACACAG-3′
HEV2m6F	+		5′-CTGTGTCGGGTGGAATGAATAACATGTC**AA**TTGCTGCGCCCATGGGTTCG-3′
HEV2m7R	−	5079–5125	5′-CAGCAAAAGACATGTTATTCATTC**G**ACCCGACACAGAATTGAATTTG-3′
HEV2m7F	+		5′-CAAATTCAATTCTGTGTCGGGT**C**GAATGAATAACATGTCTTTTGCTG-3′
HEV2m8R	−	5101–5144	5′-GGTCGCGAACCCATGGGCG**AT**GCAAAAGACATGTTATTCATTCC-3′
HEV2m8F	+		5′-GGAATGAATAACATGTCTTTTGC**AT**CGCCCATGGGTTCGCGACC-3′
HEV2m9L	−	5090–5143	5′-GGTCGCGAACCCATGGGCGC**TTGT**AAAGACATGTTATTCATTCCACCCGACACAG-3′
HEV2m9U	+		5′-CTGTGTCGGGTGGAATGAATAACATGTCTTT**ACAA**GCGCCCATGGGTTCGCGAC-3′
HEV2m59L	−	5090–5144	5′-GGTCGCGAACCCATGGGCGC**TTGT**AAAGACA**ACAA**ATTCATTCCACCCGACACAG-3′
HEV2m59U	+		5′-CTGTGTCGGGTGGAATGAAT**TTGT**TGTCTTT**ACAA**GCGCCCATGGGTTCGCGACC-3′
HEV2mGAAU	+	4655–4705	5′-CAGGTGGCTGCCTTTAAAGGTG**CC**G**CC**TCGATAGTGCTTTGCAGTGAGTAC-3′
HEV2mGAAL	−		5′-GTACTCACTGCAAAGCACTATCGA**GG**C**GG**CACCTTTAAAGGCAGCCACCTG-3′
LNAs			
p19 antisense	−	5105–5126	5′-G+CAG+CA+AA+AG+AC+ATGTT+ATT+CA-3′
p19 sense	+		5′-T+GAA+TAACA+TGT+CTTT+TG+CT+GC-3′

[a] Polarity of primers or LNAs on the HEV genome. +, forward; −, reverse.
[b] Positions of primers or LNAs on the HEV genome.
[c] Sequences of primers or LNAs. The mutated nucleotides are underlined and in boldface. The modified nucleotides in LNAs are indicated with +.

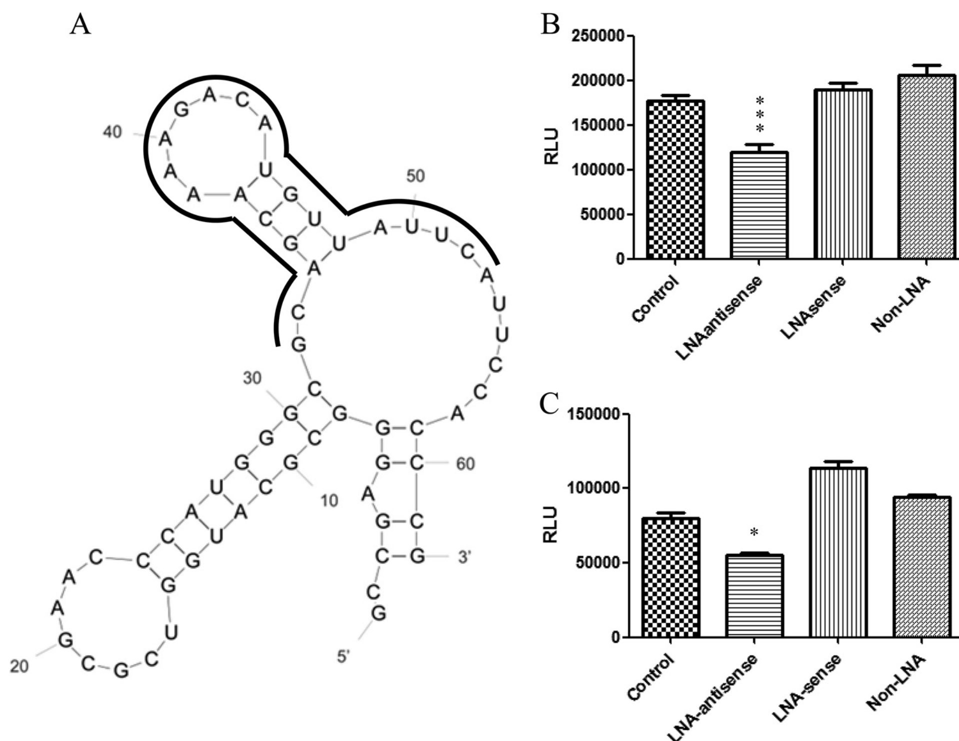

FIG. 3. Inhibition of HEV Rluc replicon replication by LNAs. (A) The position of LNA on the stem-loop structure in the junction region of the HEV genome (targeting nt 5105 through 5126) is indicated with a boldface solid line. (B) Relative luciferase activity in Huh7 S10-3 cells cotransfected at the same time with HEV Rluc replicon and LNA sense, LNA antisense, or control oligonucleotide (100 pmol per well in 24-well plate), respectively. (C) Relative luciferase activity in Huh7 S10-3 cells transfected with LNA sense, LNA antisense, or control oligonucleotide at 48 h after the transfection of the HEV Rluc replicon RNA. Data are from an average of eight separate replicate experiments, and the error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

HEV replication as efficiently as did mutant M4 that contained two additional adenosine nucleotide changes compared to M3. Even a single nucleotide mutation of the AGA motif on the loop (M1) inhibited HEV replication, suggesting that the nucleotides on the loop of SL are important for HEV replication and that the AGA motif is critical for HEV replication. The mutation on either leg of the stem (M5 and M9) also significantly inhibits HEV replication. The mutation that broke one base pair (U-G) on one leg of the stem (M2) also inhibited HEV replication, indicating that the structure of SL is also important for HEV replication. Although the HEV EGFP replicon system is not as sensitive as the Rluc system, the results with the EGFP replicon-based SL mutants are qualitatively similar to those with the Rluc-based mutants (data not shown).

It is noteworthy that the conserved AAUAAC sequence in the sense genome of the junction region, which was identified as an important motif for HEV replication *in vivo* (19), has 3 nt overlapped with one leg of the SL stem. The mutations of AAUAAC to AACAUG that resulted in less-efficient replication (19) actually broke two base pairs on the SL stem and thus may change the SL structure and inhibit HEV replication. However, we failed to rescue HEV replication by replacing the stem with a mutated complement sequence (M59) on the stem of SL (Fig. 2B), suggesting that both the sequence and structure of the SL play an important role in HEV replication. Elimination of the predicted JC virus (JCV) repeated sequence (25) and enhancer core motif (41) (M7) has no significant effect on HEV replication. Furthermore, mutations in the metal response element (MRE) motif CS2 (8) (M8) reduced HEV replication, and the combination of M7 and M8 mutations (M78) has a similar effect on HEV replication compared to that of the single mutation (M7 or M8), suggesting that these motifs may regulate but are not important for HEV replication.

LNAs targeting the SL structure inhibit HEV replication. Locked nucleic acid (LNA) bases contain a bridging methylene carbon between the 2′ and 4′ position of the ribose ring (4), and this constraint preorganizes the oligonucleotide backbone and can increase melting temperature (T_m) values by as much as 10°C per LNA substitution. Chimeric LNAs have been demonstrated not only to be active antisense agents (5, 16) but also to block the internal ribosomal entry site and inhibit translation (33). Thus, to further verify the importance of the SL on HEV replication, we designed and synthesized two LNAs that specifically target both the sense and the antisense sequences of the SL structure (Fig. 3A; Table 1). An oligonucleotide unrelated to HEV sequence, the M13 forward primer, was used as the non-LNA control. After cotransfecting each LNA with HEV Rluc replicon RNA at the same time into Huh7 cells, we measured the Rluc signal at 5 days posttransfection. The results showed that the antisense LNA inhibited Rluc signals by 42% (Fig. 3B), whereas the sense LNA has no effect on HEV replication. When the LNAs were transfected into cells at 48 h after the transfection of HEV Rluc replicon RNAs, similar inhibition results were observed with the antisense LNA (Fig. 3C). The inhibition of antisense LNA on

HEV replication may function through blocking the negative RNA from binding to the RdRp and/or another factor(s) that initiates the replication of subgenomic RNA. The signal from Huh7 cells transfected with HEV Rluc replicon and LNA sense is higher than that from cells transfected with M13 forward primer, but the difference is not statistically significant. This could be due to the variation of nonspecific inhibition of LNA sense and M13 forward primer on the replication of HEV Rluc RNA. The LNA results further confirmed that the SL sequence is important for HEV replication and that the conserved SL structure in the junction region could be a potential target for HEV drug development.

In summary, we identified the nucleotides on the SL structure of the junction region in the HEV genome that are important for HEV replication and demonstrated that both the sequence and the structure of the SL are critical for HEV replication.

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