Published in final edited form as:

J Med Virol. 2018 September ; 90(9): 1524–1531. doi:10.1002/jmv.25215.

Roles of the genomic sequence surrounding the stem-loop structure in the junction region including the 3' terminus of ORF1 in hepatitis E virus replication

Dianjun Cao, Yan-Yan Ni, Michelle Walker, Yao-Wei Huang, and Xiang-Jin Meng*

Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia

Abstract

Hepatitis E virus (HEV), a member of the family *Hepeviridae*, causes both acute and chronic viral hepatitis. We have previously demonstrated that the stem-loop structure in the junction region (JR) of HEV genome plays a critical role in HEV replication. However, the function of the sequence bordering the JR, including the 3' terminus of ORF1, in HEV replication is unknown. In this study, a panel of HEV Renilla luciferase (Rluc) replicons containing various deletions at 5' or 3' termini of the JR were constructed to determine the effect of the deletions on HEV replication in Huh7 human liver cells. We showed that even a single nucleotide deletion at the 5' terminus of the JR abolished HEV replication, whereas deletions at the 3' terminus of the JR also decreased virus replication efficiency. Furthermore, we also constructed FireFly luciferase (FFluc) and Rluc dual reporter HEV replicons containing the 3' terminal ORF1 of various lengths, and the JR inserted upstream of the Rluc reporter. A higher level of HEV replication was observed in cells transfected with replicons containing the 3' terminal ORF1 than that of the JR only replicon. We also showed that the ORF3 noncoding sequence plus the JR promoted a higher level of translation activity than that promoted by JR and ORF2 noncoding sequence.

Keywords

Hepatitis E virus (HEV); Junction region; Virus replication; Luciferase replicons; ORF1 terminus; Promoter

* Author for Correspondence: X.J. Meng, M.D., Ph.D., University Distinguished Professor, Department of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, 1981 Kraft Drive, Blacksburg, VA 24061-0913, Phone: (540) 231-6912; Fax: (540) 231-3414; xjmeng@vt.edu.

Conflicts of interest: The authors declare that they have no competing interests.

Author Contributions: D.C. designed the study, conducted all the experiments, and wrote the manuscript. Y.Y.N. and M. W. helped perform the mutagenesis and luciferase assay experiments. Y.W.H. contributed to the design of the study, and provide plasmids. X.J.M. participated in its design and coordination of the study, and wrote the manuscript. All authors read and approved the final manuscript.

Disclaimers: This paper has not been published or presented elsewhere in part or in entirety, and is not under consideration for publication by another journal.

1. Introduction

Hepatitis E virus (HEV), the causative agent of hepatitis E, infects approximately 20 million people worldwide leading to more than 56,600 deaths every year^{1,2}. Hepatitis E is an important public health disease with significant global disease burden^{3,4}. HEV typically causes a self-limiting acute viral hepatitis, however HEV infection can also cause high mortality (up to 25%) in infected pregnant women⁵ and progress into chronicity in immunocompromised individuals, such as solid organ transplant recipients⁶.

HEV, a member of the family *Hepeviridae*⁷, is a single-stranded, positive-sense, RNA virus with a genome of approximately 7.2 kb in size, which consists of 3 partially-overlapping open reading frames (ORFs)^{8–10}. The ORF1 encodes the nonstructural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a small multi-functional protein^{8,11,12}. The ORF2 and ORF3 overlap each other and are translated from a single bicistronic mRNA, but neither of them overlaps ORF1^{13,14}. Two cis-reactive elements (CRE), which are critical for HEV replication, were identified in the noncoding regions (NCR) of HEV genome. The first CRE overlaps with the 3' end of the ORF2 and the 3' NCR that binds with viral RNA-dependent RNA polymerase (RdRp)^{15–17}. The second CRE locates in the junction region (JR) between ORF1 and ORF2, and may act as the promoter for the subgenomic mRNA^{13,18}. An additional RNA element, which contains two conserved stem-loop structures, was identified in the central region of the ORF2¹⁹ and may play an important role in the early steps of virus replication.

Previously we have demonstrated that the stem-loop structure in the JR of HEV genome plays a critical role in HEV replication, although the function of the sequence neighboring the stem-loop structure in the JR is unclear²⁰. In this present study, we identified that the nucleotides at the 3' terminus of the ORF1 immediately preceding the JR is important for HEV replication. We also determined the effect of the nucleotides at the 5' and 3' of the JR on HEV replication.

2. Materials and Methods

2.1. Cell cultures and HEV replicons

The Huh7-S10-3 human liver cell line and the genotype 1 human HEV (strain Sar55) infectious clone are gifts from Dr. Suzanne U. Emerson (NIAID, NIH, Bethesda, MD). The Huh7-S10-3 cell was cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO₂ incubator. The Sar55 HEV replicons, pSKHEV2-EGFP²¹ and pSKHEV2-hRluc²⁰, were constructed previously in our laboratory.

2.2. Construction of a panel of HEV replicon mutants and *in vitro* RNA transcription of HEV replicons

We used the HEV replicon pSKHEV2-hRluc, which is a novel HEV replicon system that has the nt 5148 to 5816 of the pSK-HEV-2 infectious cDNA clone replaced with the Renilla luciferase (Rluc) gene²⁰, as the backbone to construct 9 HEV replicon mutants with QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). These HEV replicon mutants were designated as 5' 1, 5' 2, 5' 3, 5' 4, 5' 6, 5' 7, 3' 1, 3' 2, and 3' 3, respectively

(Fig. 1, Fig. 2). Six of these replicon mutants contain various deletions at the 5' terminus of the JR, and the 3 remaining replicon mutants contain different deletions at the 3' terminus of the JR (Fig. 1). The primers used for the construction of the HEV2Rluc replicon mutants were listed in Table S1. The pSK-HEV2-Rluc was used as a control.

Capped RNA transcripts from each of the 9 HEV mutant replicons as well as the wild-type HEV replicon were synthesized *in vitro* with the mMESSAGE mMACHINE T7 kit (Ambion) (10, 24, 26). Each of the capped RNA transcripts of the replicons was transfected into Huh7-S10-3 liver cells, respectively, with the DMRIE-C reagent (Invitrogen)²⁰. The luciferase activities in transfected liver cells were measured with a dual luciferase reporter assay system (Promega) at 5 days post-transfection. The Renilla luciferase signal of each sample was normalized by using the cotransfected Firefly luciferase RNA.

2.3. Construction of dual Luciferases HEV replicons

By using the pSK-HEV2-HuRluc as the template, we amplified by PCR the HuRluc and partial HEV ORF2 fusion fragment with primers HEVRluc7251U22 and HuhRluc915L43. The amplified PCR product was subsequently digested with *MfeI* and *EcoRI* restriction enzymes, and purified with the Wizard gel and PCR clean-up system (Promega). The pSK-HEV-2-FFluc replicon plasmid was digested with *EcoRI* and treated with Shrimp Alkaline Phosphatase (rSAP). The PCR product was then ligated into the linearized pSK-HEV-2-FFluc replicon to create the pSK-HEV2-FFluc/Rluc replicon that contains both FFluc and Rluc reporter genes. The pSK-HEV2-FFluc-HF-Rluc (defined as "HF") contains the 3' terminus of ORF1 and JR upstream of the FFluc reporter gene (Fig. 3). The pSK-HEV2-FFluc-JF-Rluc replicon (defined as "JF") contain JR upstream of the FFluc (Fig. 3).

To introduce different regulatory HEV genomic fragments into the dual reporter HEV replicon, the pSK-HEV2-FFluc/Rluc plasmid was digested with *SbfI* and *EcoRI* and used as a backbone. The cDNA fragments containing the JR and various fragments of the 3' terminus of ORF1 were amplified by PCR from the pSK-HEV2-HuRlu template with primers HuHRluc915L43 (reverse primer), and HEVSbfIRluc7312U, HEVSbfIRluc7322U, HEVSbfIRluc7332U, and HEVSbfIRluc7342U (forward primers) (Table S1), respectively. The amplified PCR products were digested with *SbfI* and *EcoRI*, and subsequently ligated into the pSK-HEV2-FFluc/Rluc plasmid linearized with *SbfI* and *EcoRI*. The replicons were designated as 5005, 5066, 5076, 5086, and 5096, which contains 102 nt, 41 nt, 31 nt, 21 nt, and 11 nt sequence from the 3' end of HEV ORF1 inserted upstream of the Rluc reporter, respectively (Fig. 4A).

2.4. Construction of ORF1 3' terminus- and JR-driven Rluc reporter cDNA segments

Using primers HEV2:4761U31, HEV2:4921U32, or HEV2:5055U34 as the forward primers (Table S1), we amplified three fragments of different sizes from the 3' terminus of ORF1 and the immediate downstream JR by paired with primer HEV2:408L32 (end with the ORF3 start code, designated as 4761-O3, 4921-O3, and 5055-O3) or primer HEV2:427L27 (end with the ORF2 start codon, designated as 4761-O2, 4921-O2, and 5055-O2.) (Table S1) (Fig. 6A), respectively. The PCR products, were digested with *BglII* and *Acc65I*, and then ligated into pGL4.7 vector linearized with restriction enzymes *BglII* and *Acc65I*. After

sequencing confirmation, the recombinant plasmids were used as the template for PCR amplification of the corresponding cDNAs that contain a different intergenic region of HEV fused with Rluc and flanked by a T7 promoter and poly (A) with the primer set: pGL4.7T7:1U38 and pGL4.7Pa:1046L43 (Table S1) (Fig. 6B). The PCR products were purified with the Wizard gel and PCR clean-up system (Promega). By using pGL3control plasmid as template, the firefly luciferase cDNA driven by T7 promoter was amplified with the primer set: pGL3ctrl:258U24 and pGL3ctrl:2118L24 (Table S1) and used it as an internal control. The constructed cDNA fragments were used as the template to produce reporter RNAs by *in vitro* transcription.

2.5. Construction of HEV non-coding regions-driven Rluc reporter cDNA segments

Using a promoterless Rluc reporter vector pGL4.7 as the template, primer pGL4.7Pa:1046L43 as a reverse primer and primers, T7HEV2So3, T7HEV21stSo3, and T7HEV25utr as a forward primer (Table S1), respectively, we amplified the Rluc cDNA fused with the ORF3 noncoding region (GS-ORF3, from the first nucleotide of the subgenome to the ORF3 start codon), the ORF3 noncoding region plus the JR (JS-ORF3), and the 5' noncoding region at the 5' terminus of HEV genome (5'UTR), respectively. Using the pSK-HEV2-Rluc as a template, reverse primer pGL4.7Pa:1046L43 and forward primer T7HEV2So2 or T7HEV21stSo2 were used to produce HEV ORF2 noncoding region (GS-ORF2, which ranges from the first nucleotide of the subgenome to the ORF2 start codon), and ORF2 noncoding region plus JR (JS-ORF2) fused Rluc cDNAs, respectively. The resulting cDNAs were used as templates for *in vitro* transcription of reporter RNAs.

2.6. *In vitro* transcription of HEV reporter RNAs containing different HEV noncoding regions

All cDNA fragments produced above and the *Bgl*II-linearized HEV replicons were purified with the Wizard gel and PCR clean-up system (Promega), and used as the respective template for the *in vitro* RNA transcription with the mMessage mMachine T7 Ultra kit (Ambion) as described previously²⁰. The capped RNA transcripts from each of the HEV replicons or cDNA fragments were transfected into the Huh7-S10-3 liver cells with DMRIE-C reagent (Invitrogen) by following the manufacturer's instruction. The luciferase activities in transfected cells were measured with a dual luciferase reporter assay system (Promega) at 5 days post-transfection. Firefly luciferase RNA was co-transfected with the HEV Rluc replicon RNAs to normalize the Renilla luciferase signal as described previously^{20,22}.

2.7. Analysis of the RNA secondary structure in the 3' end of the HEV ORF1

The RNA secondary structures in the last 41 and 104 nucleotides of the 3' end of the ORF1 were predicted by using the mfold program as described previously²³.

2.8. Statistical analysis

All data analyses were performed with GraphPad Prism 6 (GraphPad Software, Inc.). The comparisons between two groups were calculated with two-tailed t-test, and multiple comparisons among the experimental groups were analyzed with one-way analysis of

variance (ANOVA) followed by Kruskal-Wallis test. The $p < 0.05$ was considered significant, and $p < 0.01$ was considered highly significant.

3. Result

3.1. The nucleotides at the 5' terminus of the JR are indispensable for HEV replication

In order to determine the role of the nucleotides adjacent to the stem-loop structure of the JR in HEV replication, we constructed a panel of HEV replicon mutants that contain various deletions at the 5' or 3' terminus of the JR (Fig. 1). The results revealed that the levels of HEV replication in Huh7 human liver cells for all the Rluc replicon mutants were decreased significantly compared to that of the wild-type HEV genotype 1 Rluc replicon (Fig. 2). Even a single nucleotide deletion from the 5' terminus of the JR significantly reduced the level of HEV replication, suggesting that the nucleotides in the 5' terminus of the JR are indispensable for efficient replication of HEV. However, the deletion of the first nucleotide at the 3' terminus of the JR decreased the HEV replication level more pronouncedly than the deletions of the first 2 or 3 nucleotides at the 3' terminus (Fig. 2).

3.2. The last 21 nucleotides at the 3' terminus of the ORF1 immediately preceding the JR promote HEV replication

To further understand if the JR alone is sufficient to regulate efficient HEV replication, we examined the effect of the nucleotides at the 3' terminus of the ORF1, which locates immediately upstream of the JR, on HEV replication efficiency. Since the 3' end of the ORF1 is required for the nonstructural protein expression, thus we cannot change the nucleotides directly without affecting the expression of the nonstructural protein, as even a silent mutation might affect the efficiency of the ORF1 expression, which would possibly impact the virus replication level. Therefore, to overcome this obstacle, we constructed a dual luciferase reporter system by inserting the Firefly luciferase gene (FFluc) downstream of the Rluc gene in the pSK-HEV2-Rluc HEV replicon (Fig. 3A). By using this dual reporter gene system, we inserted a duplicated HEV JR or an HEV JR plus the 3' terminal sequence of ORF1 upstream the second reporter, without affecting the expression of the nonstructural protein. Therefore, this dual reporter system allows us to directly examine the replication efficiency of various elements upstream of the Rluc reporter.

In vitro transfection experiment in Huh7 liver cells revealed that both FFluc and Rluc reporters were functional in the HEV replicon, and that the JR plus the 3' terminal sequence of ORF1 promoted a higher level of HEV replication than the JR alone (Fig. 3B). However, compared to the upstream Rluc, the FFluc signal is too weak for us to accurately compare the replication level between the two luciferase reporters. Since the expression of Rluc is more efficient than that of FFluc in the HEV replicon system, therefore we subsequently switched the position of the two reporter genes for the study of the effect of 3' terminal sequence of ORF1 on virus replication.

Using the new replicon system, we constructed five different 3' ORF1 mutant replicons by inserting various 3' terminal fragments of ORF1 upstream of the Rluc (the second reporter) (Fig. 4A). The sizes of inserted 3' ORF1 fragments range from 11 nt to 102 nt. The

replication level of the mutant replicons, as defined as the ratio of Rluc and FFluc, was measured. The results showed that the replicon containing the 21 nt of 3' end of ORF1 promoted a significantly higher level of HEV replication than the other replicons with insertions of other 3' terminal ORF1 sequence, especially for those with 41 and 102 nt insertions (Fig. 4B). The results suggested that the 3' terminal region of the ORF1, particularly the last 21 nt, plays an important regulatory role in HEV replication.

3.3. Stem-loop (SL) structures exist in the 3' terminal sequence of the HEV ORF1

The mfold analysis of the nucleotide sequence at the 3' end of HEV ORF1 revealed that the last 102 nucleotides at the 3' end of the HEV ORF1 form three SL structures (Fig. 5A), and that even the last 41 nucleotides form an SL structure (Fig. 5B). The presence of SL's in this region suggests that nucleotide sequence at the 3' end of the HEV ORF1 may possess a regulatory function in HEV replication.

3.4. The non-coding region (NCR) of the ORF3 and the JR promote the translation more efficient than the NCR of ORF2 and the JR

To determine if the nucleotides at the 3' terminus of ORF1 effect the translation of ORF3 or ORF2 protein, we constructed 3 Rluc reporter RNAs by using reporter plasmids containing three different sizes of the ORF1 3' terminal fragments together with NCR of ORF2 or ORF3 and constructed cDNA cassettes containing T7 promoter upstream, and Rluc downstream as a reporter. The three reporter RNAs of each protein were subsequently transfected into Huh7 S10-3 liver cells to assess the level of viral protein translation (Fig. 6A). The results showed that all reporter RNAs translated the Rluc at a similar level, except for the sequence from nt 5055 to the start codon of ORF3, which promoted about 2 fold higher level of reporter RNA translation than the other sequences did. Interestingly, the ORF3 NCR and the JR promoted translation activity of the reporter RNA to a higher level as compared to the ORF2 NCR and the JR (Fig. 6C).

Additionally, we also compared the translation initiation activity of different HEV non-coding regions and found that the 5' NCR of the HEV genome promotes the translation of reporter gene most efficiently, whereas the ORF3 NCR and the JR promote higher translation level than other sequences and the JR (Fig. 6D). These results indicated that the JR or JR plus the 3' terminus of the ORF1 in HEV genome promotes the translation of the ORF3 more efficiently than that of ORF2. Also, the JR together with ORF3 NCR sequence (JS-ORF3) promoted the highest level of reporter RNA translation.

4. Discussion

The lack of an efficient cell culture system for HEV propagation greatly hindered the investigation of the mechanism of HEV replication²⁴⁻²⁶. To overcome this obstacle, we utilized the luciferase replicon system to study the mechanism of HEV replication. Previously, we have demonstrated that the stem-loop structure in the JR is critical for HEV replication, although it is unknown if the surrounding nucleotides play any role in regulating the efficiency of HEV replication²⁰. Furthermore, it is also unknown whether the nucleotides in the 3' terminal region of ORF1 that is immediately upstream of the JR play a

role in the regulation of HEV replication efficiency. Therefore, in this study, we constructed various luciferase replicons of HEV, in which different mutations or deletions were introduced, to determine the effect of nucleotide mutations or deletions in and upstream of the JR on the efficiency of HEV replication.

We demonstrated that the nucleotide sequence in the 5' terminus of the JR is indispensable for HEV replication, and that the nucleotides in the 3' terminus of the JR are also important for the efficiency of HEV replication. A major challenge for assessing the role of nucleotides in the 3' terminus of the ORF1 in virus replication is to modify the nucleotide sequence in the region without influencing the ORF1 replication and translation. In this study, we successfully constructed an HEV replicon containing dual luciferase reporters, in which the FFluc immediately downstream of the ORF1 was used to monitor the HEV replication, whereas the Rluc reporter downstream the duplicated and modified JR was used to determine the effect of mutations of this region on the HEV subgenomic RNA replication. This novel HEV replicon with dual luciferase reporters enables us to assess the effect of the different regulatory elements on the efficiency of HEV Rluc replication. We demonstrated that the JR plus the nucleotides at 3' terminus of ORF1 promoted a higher level of HEV replication than did the JR alone, and that the last 21 nt in the 3' terminus of ORF1 plus the JR promoted the highest level of HEV replication. The results suggested that the nucleotide sequence at the 3' terminus of HEV ORF1 plays an important role in replication of HEV subgenomic RNA and may act as a partial HEV subgenomic RNA promoter.

Emerson et al predicted several conservation peaks at the 3' terminus of the HEV ORF1 using the conservation statistical analysis of the synonymous sites by alignments of 185 HEV ORF1 nucleotide sequences¹⁹. The predicted conservation peaks represent RNA elements that are involved in HEV replication. Interestingly, in the present study, we found that the last 41 nucleotides at the 3' end of the ORF1 fold into a stem-loop structure (Fig. 5B), which normally appears in a typical promoter²⁷. Although this region is not the core sequence of the HEV subgenomic RNA promoter, it may act as an enhancer for the subgenomic RNA promoter. As suggested by Adkins and Kao²⁸, viral RdRp recognizes and binds to the stem-loop structure in the JR (i.e., core promoter sequence), then the upstream nucleotides at 3' terminus of the ORF1 may stabilize the binding of RdRp and minus-strand RNA to enhance HEV replication. Taken together, the results from this study suggested that the sequence at the 3' terminus of the ORF1 plays an important role in regulating HEV replication, particularly in the subgenomic RNA replication, and that the 3' terminal region of the ORF1 may be a part of the subgenomic RNA promoter.

Another interesting question regarding HEV replication is the mechanism of translational regulation of the ORF3 and ORF2 proteins. In this study, we utilized an *in vitro* expression system to identify the effect of nucleotides at the 3' terminus of the ORF1 and the JR on the translation of ORF3 and ORF2, respectively. The results showed that the JR and NCR of ORF3 promoted higher translation level of reporter RNA when compared to the JR and NCR of ORF2, suggesting that the ORF3 might be translated more efficiently than ORF2. Interestingly, when the 3' ORF1 terminal sequence was added upstream of the subgenomic start site, the translation level of reporter RNA was reduced when compared to that promoted by the JR and NCR of the ORF2 or ORF3 only. This suggested that the 3' ORF1

terminal sequence is unfavorable for the downstream expression, and that the ORF2 and ORF3 are likely translated from the subgenomic mRNA that does not contain the 3' ORF1 terminal sequence.

In summary, we identified that the nucleotides, not just the stem-loop structure, in the JR of HEV genome are important for HEV replication. We also revealed that the nucleotides at the 3' end of HEV ORF1 play an important role in the subgenomic RNA replication. Furthermore, we demonstrated that the JR and NCR of ORF3 promote the translation of ORF3 more efficiently than that of ORF2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Barbara Dryman for her technical assistance. This study is supported by grants from the National Institutes of Health (R01 AI074667, and R01 AI050611).

References

1. Rein DB, Stevens GA, Theaker J, Wittenborn JS, Wiersma ST. The global burden of hepatitis E virus genotypes 1 and 2 in 2005. *Hepatology*. 2012; 55:988–997. [PubMed: 22121109]
2. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012; 380:2095–2128. [PubMed: 23245604]
3. Hakim MS, Wang W, Bramer WM, et al. The global burden of hepatitis E outbreaks: a systematic review. *Liver Int*. 2017; 37:19–31.
4. Wen GP, Tang ZM, Wang SL, et al. Classification of human and zoonotic group hepatitis E virus (HEV) using antigen detection. *Appl Microbiol Biotechnol*. 2017; 101:8585–8594. [PubMed: 29038976]
5. Meng XJ. Recent advances in Hepatitis E virus. *J Viral Hepat*. 2010; 17:153–161. [PubMed: 20040046]
6. Kamar N, Abravanel F, Lhomme S, Rostaing L, Izopet J. Hepatitis E virus: Chronic infection, extra-hepatic manifestations, and treatment. *Clinics and research in hepatology and gastroenterology*. 2015; 39:20–27. [PubMed: 25150374]
7. Smith DB, Simmonds P, et al. International Committee on Taxonomy of Viruses Hepeviridae Study G. Consensus proposals for classification of the family Hepeviridae. *J Gen Virol*. 2014; 95:2223–2232. [PubMed: 24989172]
8. Emerson SU, Purcell RH. Hepatitis E Virus. In: Knipe DM, Howley PM, editors *Fields virology* 5. Philadelphia, Pa: Lippincott-Raven Publishers; 2007 30483059
9. Ahmad I, Holla RP, Jameel S. Molecular virology of hepatitis E virus. *Virus research*. 2011; 161:47–58. [PubMed: 21345356]
10. Van der Poel WHM, Dalton HR, Johne R, et al. Knowledge gaps and research priorities in the prevention and control of hepatitis E virus infection. *Transboundary and emerging diseases*. 2018
11. Cao D, Meng XJ. Molecular biology and replication of hepatitis E virus. *Emerg Microbes Infect*. 2012; 1:e17. [PubMed: 26038426]
12. Purdy MA, Lara J, Khudiyakov YE. The hepatitis E virus polyproline region is involved in viral adaptation. *PLoS One*. 2012; 7:e35974. [PubMed: 22545153]
13. Graff J, Torian U, Nguyen H, Emerson SU. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. *J Virol*. 2006; 80:5919–5926. [PubMed: 16731930]

14. Huang YW, Opriessnig T, Halbur PG, Meng XJ. Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. *J Virol.* 2007; 81:3018–3026. [PubMed: 17202216]
15. Emerson SU, Zhang M, Meng XJ, et al. Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. *Proc Natl Acad Sci U S A.* 2001; 98:15270–15275. [PubMed: 11742081]
16. Mahilkar S, Paingankar MS, Lole KS. Hepatitis E virus RNA-dependent RNA polymerase: RNA template specificities, recruitment and synthesis. *J Gen Virol.* 2016; 97:2231–2242. [PubMed: 27324050]
17. Agrawal S, Gupta D, Panda SK. The 3' end of hepatitis E virus (HEV) genome binds specifically to the viral RNA-dependent RNA polymerase (RdRp). *Virology.* 2001; 282:87–101. [PubMed: 11259193]
18. Graff J, Nguyen H, Yu C, et al. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. *J Virol.* 2005; 79:6680–6689. [PubMed: 15890906]
19. Emerson SU, Nguyen HT, Torian U, Mather K, Firth AE. An essential RNA element resides in a central region of hepatitis E virus ORF2. *J Gen Virol.* 2013; 94:1468–1476. [PubMed: 23515023]
20. Cao D, Huang YW, Meng XJ. The nucleotides on the stem-loop RNA structure in the junction region of the hepatitis E virus genome are critical for virus replication. *J Virol.* 2010; 84:13040–13044. [PubMed: 20943962]
21. Emerson SU, Nguyen H, Graff J, Stephany DA, Brockington A, Purcell RH. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J Virol.* 2004; 78:4838–4846. [PubMed: 15078965]
22. Pudupakam RS, Huang YW, Opriessnig T, Halbur PG, Pierson FW, Meng XJ. Deletions of the hypervariable region (HVR) in open reading frame 1 of hepatitis E virus do not abolish virus infectivity: evidence for attenuation of HVR deletion mutants in vivo. *J Virol.* 2009; 83:384–395. [PubMed: 18945785]
23. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic acids research.* 2003; 31:3406–3415. [PubMed: 12824337]
24. Schemmerer M, Apelt S, Trojnar E, Ulrich RG, Wenzel JJ, Johne R. Enhanced Replication of Hepatitis E Virus Strain 47832c in an A549-Derived Subclonal Cell Line. *Viruses.* 2016;8.
25. Okamoto H. Hepatitis E virus cell culture models. *Virus research.* 2011; 161:65–77. [PubMed: 21316402]
26. Takahashi H, Tanaka T, Jirintai S, et al. A549 and PLC/PRF/5 cells can support the efficient propagation of swine and wild boar hepatitis E virus (HEV) strains: demonstration of HEV infectivity of porcine liver sold as food. *Arch Virol.* 2012; 157:235–246. [PubMed: 22048607]
27. Sztuba-Solinska J, Stollar V, Bujarski JJ. Subgenomic messenger RNAs: mastering regulation of (+)-strand RNA virus life cycle. *Virology.* 2011; 412:245–255. [PubMed: 21377709]
28. Adkins S, Kao CC. Subgenomic RNA promoters dictate the mode of recognition by bromoviral RNA-dependent RNA polymerases. *Virology.* 1998; 252:1–8. [PubMed: 9875310]

Fig. 1.

Genomic organization of the HEV replicon depicting the position of the junction region (JR) containing the various deletions. The sequence, shown as the positive polarity of HEV genome, extends from nt position 5100 through 5133. The stop codon TGA of ORF1 was shown in boldface. The HEV subgenome start site was indicated with an arrow, and the first two nucleotides were bolded. "X" indicated the deletion of nucleotide(s).

Fig. 2.

The replication level of various HEV Rluc replicons containing different modified JR. HEV2: HEV2Rluc replicon; 5 Δ 1, 5 Δ 2, 5 Δ 3, 5 Δ 4, 5 Δ 6 and 5 Δ 7: HEV2Rluc replicons containing 1, 2, 3, 4, 6 and 7 nt deletion at the 5' terminus of the JR, respectively; 3 Δ 1, 3 Δ 2, and 3 Δ 3: HEV2Rluc replicons containing 1, 2, and 3 nt deletions at 3' end of the JR. Data represent an average of 8 separate replicas, and the error bars indicate standard deviation (SD). The relative luciferase activities were measured and normalized with signals of cotransfected Firefly luciferase RNA at 5 days post-transfection. The differences of the signals produced by HEV Rluc mutants and wild-type Rluc replicon were compared by one-way ANOVA using Kruskal-Wallis test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 3. Construction of dual luciferase replicons of HEV. **(A)** A schematic representation of HEV genome and dual luciferases replicons containing the JR or the JR plus the 3' terminus of ORF1 upstream the second luciferase reporter, FFLuc. Numbers flank report(s) indicate the nt position in the Sar-55 genome. EcoRI is the insertion (truncation) site in the ORF2. **(B)** Luciferase activity in Huh7 S10-3 liver cells at 5 days after the transfection of the HEV dual luciferase replicon RNA containing the JR and 3' terminus of the ORF1 or the JR only upstream the second reporter, FFLuc. HF: indicated the insertion of HEV 3' end of ORF1 and JR upstream of FFLuc. JF: indicated insertion of JR upstream of FFLuc. Data represent an average of 8 separate replicas, and the error bars indicate SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 4.

Comparison of the replication level of HEV dual luciferase replicons containing different lengths of 3' terminal ORF1 fragment and the JR upstream the Rluc. **(A)** A schematic representation of HEV genome and dual luciferases replicons. The fragment size in nucleotides (nt) and the start site of 3' terminus of ORF1 were indicated. Numbers flank report(s) indicate the nt position in the Sar-55 genome. EcoRI is the insertion (truncation) site in the ORF2. **(B)** Luciferase activity in Huh7 S10-3 liver cells at 5 days after the transfection of the HEV dual luciferase replicon RNA containing the JR and different lengths of the 3' terminal ORF1 fragment upstream the second reporter, Rluc. 5096, 5086, 5076, 5066, and 5005 indicated the start nucleotide location of 3' terminal sequence of HEV ORF1 fragments, which corresponds to the insertion size of 11, 21, 31, 41, and 102 nt, upstream of gene Rluc reporter, respectively. Data represent an average of 8 separate replicas, and the error bars indicate SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 6.

In vitro translation of Rluc RNA promoted by different potential regulatory elements of HEV. **(A)** A schematic representation of the different lengths of fragments from the 3' terminus of HEV ORF1 and the JR. The numbers indicated the start site of different fragments. **(B)** A schematic representation of Rluc reporter RNA driven by different lengths of the 3' terminus of the HEV ORF1 plus the JR. JR: junction region. **(C)** The translation level of Rluc promoted by different lengths of the 3' terminus of the HEV ORF1 in Huh7 S10-3 liver cells. 4761-O2, 4921-O2, and 5055-O2 indicated that the reporter RNAs had nucleotide sequence of 3' ORF1 terminus from nt position 4761, 4921, and 5055 of the HEV genome that were inserted together with NCR of HEV ORF2 upstream of the reporter Rluc. 4761-O3, 4921-O3, and 5055-O3 indicated that the reporter RNA had nucleotide sequence of 3' ORF1 terminus from nt position 4761, 4921, and 5055 of HEV genome that were inserted together with NCR of HEV ORF3 upstream of the reporter Rluc. **(D)** The translation level of Rluc promoted by different elements in Huh7 S10-3 liver cells. 5' UTR: the NCR of 5' end of HEV genome was inserted upstream of the reporter RNA; JS-ORF2: the sequence from JR to the start codon of ORF2 was inserted upstream the reporter RNA; GS-ORF2: insertion of sequence from the start of subgenome to the start codon of ORF2 upstream the reporter RNA; JS-ORF3: insertion of sequence from the JR to the start codon of ORF3 upstream the reporter RNA; GS-ORF3: insertion of sequence from the start of subgenome to the start codon of ORF2 upstream the reporter RNA. Data represent an average of eight separate replicas, and the error bars indicate SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.