

**Understanding the Role of the Hypervariable Region in the Open Reading
Frame 1 of the Hepatitis E virus in Viral Replication**

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in
partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

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January 21, 2011

Blacksburg, VA

Keywords and abbreviations: hepatitis E, hepatitis E virus, HEV, human HEV, swine HEV,
avian HEV, big liver and spleen disease, BLSD, hepatitis–splenomegaly syndrome, HS
syndrome, hypervariable region, HVR, subgenomic replicon

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Abstract

Hepatitis E virus (HEV) is a major cause of enterically transmitted acute viral hepatitis in developing countries that lack proper hygienic infrastructure. Hepatitis E is globally distributed and has emerged as an important public health disease in both developing and industrialized countries. HEV is a non-enveloped virus carrying a single-stranded positive-sense RNA genome of approximately 7.200 bp in length. The life cycle of HEV is poorly understood due to the lack of an efficient cell culture system. Animal model systems, including non-human primates, swine, and chickens are being used to study some fundamental aspects of the HEV biology. Recently, novel animal strains of rat and rabbit HEV have been discovered, and whose usage as animal model systems needs to be established. HEV infections in pigs and chickens provide excellent model systems to study the replication and pathogenesis aspects of HEV. Recently, we identified a hypervariable region (HVR) in the open reading frame 1 (ORF1) of HEV. The objectives of this dissertation were to utilize chicken and swine model systems to study the role of HVR in HEV infection *in vivo*, to determine the effects of HVR on replication of HEV *in vitro*, and to analyze the effect of exchange of HVR among different genotypes on the replication-competency and virion production *in vitro*.

Extensive sequence variability in the HVR among HEV strains of different genotypes prompted us to study the dispensability of this region. Initially we constructed two partial deletion mutants of genotype 1 human HEV, hHVRd1 and hHVRd2, with in-frame deletion of amino acids (aa) 711 to 777 and 747 to 761 in the HVR of a sub-genomic GFP HEV replicon. Expression of enhanced green fluorescent protein by the mutant hHVRd2 confirmed the dispensability of amino acid residues 747-761 of the HVR. To confirm our *in vitro* results, specific-pathogen-free (SPF) chickens were intra-hepatically inoculated with capped RNA transcripts from three avian HEV HVR-deletion mutants: mutants aHVRd1 (Δ 557-585), aHVRd2 (Δ 612-641), and aHVRd3 (Δ 557-641). Chickens intra-hepatically inoculated with the

mutants, aHVRd1 and aHVRd2, developed active viral infection as evidenced by seroconversion, viremia, and fecal virus shedding. Mutant aHVRd3, with a larger HVR deletion, was apparently attenuated in chickens. Additionally, we used the swine model system to further verify our results from the chicken study. The infectivity of four genotype 3 swine HEV HVR-deletion mutants, sHVRd1 (Δ 712-790), sHVRd2 (Δ 722-781), sHVRd3 (Δ 735-765), and sHVRd4 (Δ 712-765) constructed using the genotype 3 swine HEV as the backbone was determined in SPF pigs. Pigs intra-hepatically inoculated with capped RNA transcripts from the mutants sHVRd2, sHVRd3, and sHVRd4 developed active viral infection, whereas mutant sHVRd1 (Δ 712-790), with a nearly complete HVR deletion, exhibited an attenuation phenotype. The data from these studies indicate that deletions in HVR do not abolish HEV infectivity *in vitro* or *in vivo*, although evidence for attenuation was observed for HEV mutants with a larger or nearly complete HVR deletion.

To further elucidate the role of HVR in HEV replication, we investigated the effects of serial amino acid deletions in HVR on the replication of HEV. We first constructed a genotype 1 human HEV luciferase replicon by replacing the ORF2 gene that encodes for the capsid protein with the fire fly luciferase reporter gene. Using the backbone of human HEV genotype 1 luciferase replicon, we constructed a series of HVR-deletion mutants with deletions of variable lengths in the HVR. Amino acid deletions Δ 711-725, 711-740 and Δ 711-750 were engineered at the N-terminus, deletions Δ 729-754, Δ 721-766, and Δ 716-771 were engineered in the central region, and deletions Δ 761-775, Δ 746-775, and Δ 736-775 were engineered at C-terminus of the HVR. The effects of these serial deletions on HEV RNA replication in the human liver carcinoma cell line, Huh7, were examined. Replication levels of mutants carrying these deletions were compared with that of the wild-type HEV in Huh7 cells. We observed that deletions in the HVR did not abolish viral RNA synthesis but substantially reduced the replication levels of viral RNA, as measured by the reporter luciferase activity. To further verify the effects of HVR deletions on viral RNA replication as observed with the genotype 1 human HEV replicon, we subsequently used a genetically-distinct strain of HEV, avian HEV, and constructed an avian HEV sub-genomic luciferase replicon by substituting the ORF2 gene of avian HEV with the *fire fly* luciferase gene. Avian HEV HVR-deletion mutants Δ 557-603, Δ 566-595, and Δ 573-587 were then engineered using the backbone of avian HEV luciferase replicon. The replication efficiency of the three deletion mutants of avian HEV in chicken liver hepatoma cell line, LMH, was

evaluated. Compared with the wild-type avian HEV, the viral RNA synthesis of the avian HEV HVR-deletion mutants was considerably reduced by the HVR deletions. To analyze the impact of the complete HVR deletion on avian HEV infectivity, we constructed an avian HEV mutant with a deletion of the entire HVR region (aa Δ 557-603) using the avian HEV infectious cDNA clone as the backbone. After confirming the viability of the complete HVR-deletion mutant in LMH cells, SPF chickens were intrahepatically inoculated with capped RNA transcripts generated from the mutant. None of the chickens inoculated with the complete HVR-deletion mutant showed evidence of HEV infection, indicating that drastic reduction in replication levels due to complete HVR deletion has resulted in the loss of virus infectivity. The results indicated that HVR may have critical residues that may interact with viral/and or host factors and modulate the replication efficiency of HEV.

In the final part of the dissertation research, we sought to determine if the variable sequences of HVR are genotype-specific for *in vitro* virus replication. By using the genotype 1 human HEV as the backbone, we swapped the HVR of genotype 1 human HEV with the HVRs of the genotype 3 swine HEV and the distantly-related avian HEV to construct two inter-genotypic chimeras, pSKHEV2-Sw and pSKHEV2-Av. Similarly, by using the genotype 3 swine HEV as the backbone, the HVR of genotype 3 swine HEV was swapped with the HVR of genotype 1 human HEV to construct the chimera, pSHEV3-Hu. The viability of these chimeras was tested in Huh7 cells that are permissive for HEV replication. Immunofluorescence assay (IFA) with anti-HEV antibodies revealed that all the three chimeras were replication-competent in Huh7 cells. The infectivity of these chimeras was subsequently evaluated in HepG2 cells. The results showed that exchange of the HVR between different genotypes of mammalian HEVs does not abolish the replication competency and infectivity of HEV. This finding suggests that HVR is not genotype-specific with respect to viral replication and infectivity. The absence of detectable viral antigen in HepG2 cells infected with chimera pSKHEV2-Av suggested a functional incompatibility of the HVR of avian HEV in the mammalian HEV genome.

In summary, we identified a highly variable sequence, HVR, in the ORF1 of the HEV genome, and the sequences of the HVR vary significantly among HEV strains of different genotypes. We found that the HVR contain sequences that are dispensable for virus infectivity both *in vitro* and *in vivo*. Deletion analysis of HVR revealed that the region may play a role in modulating the replication efficiency of HEV RNA by interacting with viral and/or host factors.

Finally, we demonstrated that HVR is not genotype-specific for virus replication and the region can be functionally replaced between mammalian HEV genotypes for virus replication and virion production *in vitro*. The results from this dissertation research have important implications for better understanding the biology and mechanism of HEV replication and may aid in our efforts to eventually develop a modified live-attenuated vaccine against HEV.

Dedication

To my parents Raghavendra Rao Naidu and Prabhavathi, sister Kavitha and brother Meghanath.

Acknowledgements

The successful completion of this research is obviously not possible without the personal and practical support of numerous sources and I wish to acknowledge them all.

First, I would like to express my deepest gratitude to my advisor, Dr. Xiang-Jin Meng, for his excellent guidance, encouragement, support, patience, and most importantly for providing me the opportunity to work with him.

It is an honor for me to thank the members of my doctoral committee, Dr. Roger J. Avery, Dr. Stephen M. Boyle, Dr. Kevin Myles, and Dr. Nammalwar Sriranganathan for their valuable suggestions, insightful discussions, and support. I would like to thank Dr. Patrick Halbur and Dr. Tanja Opriessnig for performing the critical animal studies described in this dissertation. I am thankful to Dr. Pierson and Dr. LeRoith for their help with the chicken experiments.

I would like to thank all my colleagues of the molecular virology lab: Alicia Feagins, Brent Sanford, Yanyan Ni, Nathan Beach, Dianjun Cao, Nicole Plaskon, Caitlin, and Sara Smith for their scientific feedback. I appreciate the technical help of Scott Kenney, Barbara Dryman, Laura Cordoba, Yao-Wei Huang and Padma Billam during my research studies. I acknowledge the services of animal care staff, Pete Jobst, Steve Salmon, and Pamela Mohr.

My parents and siblings, Raghavendra, Prabhavathi, Kavitha and Meghanath, receive my deepest gratitude and love for their unwavering support and well wishes during the entire period of my studies. I also acknowledge the support I received from all the respondents in the area of my study, my friends, colleagues and all those people not mentioned here whose invisible hands facilitated successful completion of this research.

This research was supported by grants from National Institutes of Health (AI050611, AI065546 and AI074667).

Attribution

This dissertation is composed of an introduction, conclusion, and four Chapters. These four Chapters are written in journal format as all will be submitted to archival journals for publication. The following individuals serve as co-authors on one or more Chapters.

Dr. Xiang-Jin Meng- MD, PhD (Professor of Molecular Virology, VA-MD Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University) is the primary Advisor and Committee Chair. Prof. Meng provided excellent guidance during my research in understanding the role of hypervariable region (HVR) of hepatitis E virus (HEV) in replication. Furthermore, Prof. Meng also helped me in writing dissertation by providing multiple insightful comments and suggestions.

Dr. Yao-Wei Huang is a research scientist in the author's group. He provided considerable insight on the topics presented in the dissertation. He is a Co-author of the Chapters 2, 3 and 4.

Dr. Patrick Halbur and **Dr. Tanja Opriessnig** (College of Veterinary Medicine, Iowa State University) worked in collaboration with the author to perform critical animal studies described in chapter 2 of the dissertation.

Dr. F. William Pierson- Ph.D. (Hospital Director, Veterinary Medicine Clinical Services, VA-MD Regional College of Veterinary Medicine) contributed to Chapters 2 and 3 of the dissertation with his expertise in inoculating chickens. Dr. Pierson is a Co-author of the Chapters 2 and 3.

Dr. Scott P. Kenney is a Senior Research Associate in author's group. He has contributed to Chapter 3 of the dissertation by helping the author to collect clinical samples from chickens. Dr. Kenney is a Co-author of Chapter 3

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Dr. Laura Cordoba Garcia is a Senior Research Associate in author's group. She performed critical in vitro studies described in Chapter 4 of the dissertation. Dr. L. Córdoba is a Co-author of Chapter 4.

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General Introduction

Hepatitis E virus (HEV) is the etiological agent of hepatitis E, one of the leading causes of acute viral hepatitis that does not develop into chronicity in normal individuals. HEV is primarily transmitted by the fecal-oral route through contaminated drinking water and food supplies. The disease is globally distributed, with large epidemics occurring in developing countries lacking proper sanitation systems (11, 31). Industrialized countries like United States (US), Europe (Eu), and Japan also witnessed sporadic cases of hepatitis E, supporting the ubiquitous nature of HEV (81). Young adults within the age group 15-40 are commonly affected with relatively low mortality rates of about 1% (31). The disease is more severe in pregnant women, especially in the last trimester of pregnancy. The mortality rates in pregnant women may reach up to 25 %(69).

Though hepatitis E is sporadic in the United States, relatively high seroprevalance HEV has been recorded (40, 87). HEV antibodies were identified in many animal species, including horses, sheep, goat, mongoose, wild-boars, cats, dogs, rats, rabbits, cattle, and deer(56, 89, 100, 104, 121, 139, 140). Swine HEV in pigs, was the first animal strain of HEV that has been discovered, followed by avian HEV in chickens (48, 86). Recently, novel strains of HEV have also been identified from wild boar, sika deer, mongoose, rats, and rabbits (48, 56, 90, 110, 112, 140). Furthermore, interspecies transmission of HEV was experimentally demonstrated (35, 46, 84). These findings clearly indicate that animal reservoirs exist for HEV, and that the disease is zoonotic. Recently, sporadic cases of zoonotic transmission of hepatitis E have been documented (120, 137). The facts noted above clearly highlight hepatitis E as an important public health concern.

A major impediment in HEV research has been the lack of cell culture systems supporting virus production. This obstacle was overcome by the recent development of infectious cDNA clones of HEV that allow production of viruses infectious both *in vitro* and *in vivo* (32, 50, 52). In the absence of efficient cell culture systems, HEV infections in pigs and chickens provide alternative model systems to study replication and pathogenesis of HEV. Subgenomic replicons derived from the infectious cDNA clones of HEV will allow us to unravel some of the fundamental aspects of HEV replication in cell cultures.

Though open reading frame 1 (ORF1) of HEV has elements necessary for replication, it contains a hypervariable region (HVR), which is highly heterogeneous and variable. The size differences in HEV genomes are confined almost exclusively to the HVR of the ORF1. The role of a particular motif in the functional activity of viral protein can be identified by deleting it and investigating the effect of the deletion on the function of the protein. Recently established reverse genetic systems for HEV and animal model systems permit us to examine the dispensability of amino acid sequences in HVR for HEV infectivity *in vitro* and *in vivo*. Construction of luciferase replicon systems for genotype 1 human HEV and avian HEV will allow us to effectively quantify viral replication *in vitro*. Furthermore, these replicon systems will facilitate analysis of the effects of HVR deletions on HEV replication *in vitro*. It is important to determine if HVR has genotype-specific functions by swapping HVRs belonging to different genotypes of HEV. These studies will give us a better understanding of the possible viral determinants that govern HEV replication and/or infectivity.

Chapter 1

Literature Review

Hepatitis E disease

Hepatitis E, previously known as enterically transmitted non-A, non-B hepatitis (ET-NANB)(68, 147), is a common cause of acute hepatitis in humans. Hepatitis E is an emerging infectious disease with a worldwide distribution. Hepatitis E virus (HEV), the causative agent of hepatitis E, is responsible for large waterborne epidemics in many developing countries of Asia, Africa and North America (12, 35). Sporadic cases of hepatitis E have also been reported from industrialized countries (89). Young adults between the ages of 15-40 are mainly affected with relatively low mortality rate of about 1% (35). The most peculiar aspect of the disease is its increased severity in infected pregnant women, with mortality rates reaching up to 15-25% (76).

History of Hepatitis E virus (HEV)

A large epidemic of water-borne acute viral hepatitis in New Delhi in 1955 to 1956 was retrospectively found to be associated with a new viral etiological agent that was provisionally named as non-A, non-B hepatitis virus(12). Spherical, 27-30 nm, virus-like particles were identified using immune electron microscopy to examine the feces of a volunteer who ingested fecal matter from patients with non-A, non-B hepatitis(12). The genome of the new etiological agent was cloned in 1990, and the virus was named as hepatitis E virus (HEV)(112). The entire genome of HEV was sequenced in 1991(129).

Taxonomy and Classification

HEV was initially classified in the family *Caliciviridae* on the basis of similar morphology, physiochemical characteristics and superficial resemblance in genomic organization to caliciviruses. However, the lack of significant sequence homology and presence of major differences in its genomic organization from that of caliciviruses resulted in reclassification of HEV (16). HEV has recently been classified in a sole genus *Hepevirus* of the *Hepeviridae* family.

The data accumulated so far based on sequence and phylogenetic analysis suggest the presence of at least four major geographically distinct HEV genotypes in mammalian species:

genotype 1 (human HEV strains from Asia, Africa, and a recently identified strain from Cuba) (8, 129, 136, 143), genotype 2 (A Mexican strain of human HEV and a few variants of human HEV from Africa) (23, 54, 85, 102), genotype 3 (Strains from sporadic cases of human HEV in industrialized countries, swine HEV strains from both developing and industrialized countries, HEV strains from deer, mongoose, rabbits) (89), and genotype 4 (human and swine HEV strains of Asia mainly distributed in China, Japan, Taiwan and Vietnam) (28, 127, 145, 152). All the four genotypes of HEV share at least one immunodominant neutralization epitope and apparently comprise a single serotype (31, 160). Genotype 1 HEV, which was previously believed to infect only humans, was reportedly detected from a pig in Cambodia (24), although independent confirmation of this finding is still lacking. Avian HEV, which shares only 50 % nucleotide sequence identity with the mammalian strains, is classified as a separate species in the genus *Hepevirus*. Recently, two new HEV strains have been identified from rabbits and rats, respectively (62, 157). Based on phylogenetic analysis, the rabbit HEV appears to be a distant member of genotype 3, whereas the rat HEV may represent a new genotype (61, 62).

Epidemiology

Hepatitis E is endemic in many developing countries, particularly in many parts of Asia, Africa and in Mexico in which epidemic outbreaks have been reported. However, the disease is no longer confined to developing countries, and an increased incidence of sporadic cases of hepatitis E has been reported in industrialized countries including the US, Japan and many countries in Eu. The clinical illness is more common in adolescents and young adults. Incubation periods of 4 to 5 weeks in orally exposed volunteers (29) and of 2 to 10 weeks during hepatitis E outbreaks have been reported. The seroprevalence of HEV is age-dependent and appears to increase with age. In endemic countries, where outbreaks of hepatitis E are common, prevalence of age-specific anti-HEV antibodies is relatively low (10, 11). Antibodies to HEV are typically not detected until early adulthood, but high seroprevalence rates of more than 70% in young adults have been reported in developing countries like Egypt (41), where disease outbreaks are infrequent. In some industrialized countries where sporadic cases are reported, higher than expected prevalence rates of HEV antibodies have been documented. Surprisingly, up to 20% of normal blood donors in the US, and 10.9% of the swine workers in the state of North Carolina have tested seropositive for HEV antibodies (45). States that are large producers of swine have

the highest prevalence rates (95). Seroprevalence rates of 1.9-14.1% in healthy individuals were reported in Japan (42). Possible exposure to animal strains of HEV may be responsible for the seropositivity in healthy populations of industrialized countries.

Four routes of HEV transmission have been reported: (i) Fecal-oral transmission due to drinking of contaminated water supplies, (ii) Food-borne transmission, (iii) Vertical transmission (mother-to-child), and (iv) Parenteral blood-borne transmission. Of these, the fecal-oral route of transmission through contaminated water is the most common mode. HEV epidemics are water-borne in developing countries where proper sanitized sewage and water systems are inadequate (1, 7, 35). Zoonotic food-borne transmission of HEV has been reported in patients who consumed raw or undercooked wild boar or deer meat (87, 125, 131-133). Contamination of pig livers sold from local grocery stores with HEV was reported in Japan (153) and the US (39). Although, vertical transmission from mother to child is considered a minor route of transmission, cases have been reported during the third trimester of pregnancy with significant perinatal morbidity and mortality (70, 77, 120). The possibility of parenteral transmission of HEV has also been reported (42, 71, 86). Some reports have suggested that person-to-person transmission is uncommon (2, 69) and but possible (75, 144). Asymptomatic or subclinical HEV infections, relatively longer incubation periods, and relatively less understood reservoirs and vehicles for transmission complicate the understanding of familial transmission and thus, the understanding of dynamics of disease transmission (128).

Clinical features and pathogenesis

Hepatitis E is usually an acute self-limiting disease that does not develop into chronic infection in immunocompetent humans. The disease ranges from subclinical presentation to fulminant hepatitis (35). Persistent HEV infection, with chronic hepatitis and cirrhosis, has been reported in immunocompromised patients (44, 64). The symptoms of infected individuals are typical of acute viral hepatitis, and include jaundice, dark urine, hepatomegaly, abdominal pain, anorexia, and tenderness accompanied by nausea, vomiting and fever (45, 121). Hepatitis E clinical symptoms depend on the dose of viral exposure (139). While hepatitis E causes relatively low mortality rates (1%-4%) in general population, the disease is more severe in pregnant women, especially during the third trimester of pregnancy(109). HEV has an interesting disease course in some developing countries. The incidence of HEV infections is high

in pregnant women in some developing countries, with mortality rates of 30%-100% (101). Differences in hormonal factors and their associated effects on the immune system have been implicated in the apparent enhanced severity of the disease among pregnant women in endemic regions (101, 106). The occurrence of different genotypes and subtypes of HEV in different geographical locations, as well as variation in genetic susceptibility might explain the differences in mortality rates among pregnant women in various geographical locations (101).

Patterns of viremia and fecal shedding in HEV-infected humans are somewhat different from that of experimentally infected chickens and pigs. After fecal-oral transmission of HEV in humans, initial replication occurs in the liver, which is the main target organ (137). The development of viremia is followed by shedding of virus in the feces. Large quantities of virus are detected in the bile and presumably HEV reaches the intestines through the bile duct. In chickens experimentally infected via the oral route (20), and pigs infected by intravenous route(51), fecal shedding precedes viremia. It was shown that initial replication of HEV occurs in gastrointestinal tissues and the virus reaches the liver through the blood stream. Extrahepatic sites of HEV replication have been detected in both chickens (19) and pigs (146).

HEV viremia is transient and fecal shedding usually occurs for duration of 3-4 weeks after infection, just before the onset of clinical signs. Liver enzymes like alanine aminotransferase (ALT) are elevated in the serum during HEV infection (35, 137). HEV IgM antibodies can be detected up to 3 months (29). This followed by IgG antibodies that may last up to several months or even years after infection (6, 45). Microscopic lesions like focal necrosis and inflammation in liver are commonly observed during HEV infections. The coincidence of the occurrence of pathological lesions in the liver, and elevated levels of ALT in serum with seroconversion, suggest that hepatic injury may be immune-mediated (122).

Prevention and Control

In humans, passive transfer of low-titered serum immunoglobulins failed to prevent person-to-person transmission of HEV (69). However, high titers of anti-HEV serum immunoglobulins conferred protection to non-human primates challenged with a high dose of HEV(138), suggesting that vaccination to induce humoral immunity may be effective. Although many different HEV isolates have been identified, classification as a single serotype suggests development of a broadly protective vaccine (118). Commercial vaccines for HEV are not yet

available. The development of vaccines is hindered by the lack of an efficient cell culture system for HEV. Expression of recombinant capsid proteins has been adapted as an alternative strategy for the development of vaccines of hepatitis E. Vaccines based on recombinant capsid proteins expressed have been shown to provide protection against the disease (36), with the best candidate being a 56 kDa recombinant protein (aa 112-607) expressed in insect cells from a baculovirus vector(110). Recently, a group from China developed a vaccine named HEV 239 or Helicon that contains a truncated HEV capsid protein (aa 376-606) expressed in *Escherichia coli* (*E. coli*) (79). The vaccine was shown to be effective in the prevention of hepatitis E in the general population in China (161).

As HEV is primarily transmitted through the fecal-oral route, measures should be taken to provide safe drinking water, for proper disposal of human excreta and to practice personal hygiene. Consumption of undercooked meat and meat products should be avoided, and proper food-handling practices need to be implemented to prevent infection.

Molecular biology of hepatitis E virus

The genome of HEV is a single-stranded, positive-sense RNA of approximately 7.2 kb in length (Figure 1.1). It consists of a short noncoding region, three open reading frames (ORFs), a short [27 to 35-nucleotide (nt)] untranslated region at its 5' end (5' UTR), and a long [65 to 74 nt] 3' untranslated region (3' UTR) terminated by a poly (A) tract (35, 48, 129). ORF2 overlaps ORF3 but neither overlaps ORF1 (59). The ORF1 at the 5'-end of the genome encodes a nonstructural polyprotein with putative functional domains (Figure 1.1), including methyltransferase, Papain-like cysteine protease (PCP), helicase, and RNA-dependent RNA polymerase (RdRp) involved in HEV replication(73). The 5' end of the HEV genome is capped (155), and the guanylyltransferase and methyltransferase enzyme activities required to synthesize a 7mG cap structure are encoded by ORF1 (84). The cap structure is absolutely required for the infectivity of HEV *in vivo* (38). The RdRp activity, which is required for the replication of HEV, has been demonstrated (3). A recent study has identified a hypervariable region (HVR) in the ORF1 of HEV. It was demonstrated that HEV tolerated deletions in the HVR for its infectivity, and suggested the dispensability of the amino acid residues of this region (108). Attempts were made to study the polyprotein processing of ORF1. The processing of the ORF1 polyprotein was not observed when expressed individually in either a cell free translation system, HepG2 cells or

a vaccinia virus-driven transient expression system (5, 115). It was speculated that the papain-like cysteine protease (PCP) in the individually expressed ORF1 polyprotein is nonfunctional (115). The role of cysteine proteases in ORF1 polyprotein processing was demonstrated using a baculovirus expression system. It is not clear if the protease activity is of viral or cellular origin(119). The GDD motif found in RdRp is highly conserved (72), and mutation of the conserved GDD motif to GAD, will inactivate the polymerase and hamper HEV replication (32, 46). The putative RNA helicase contains conserved segments typical of the SF-1 helicase superfamily, which is widespread among positive-stranded RNA viruses. The biochemical characterization of the HEV helicase revealed the NTPase and RNA duplex-unwinding activities of the protein(66). Helicase-associated RNA 5' -triphosphatase activity involved in 5' cap formation has also been demonstrated for HEV (67). The possible role of the 3' end of HEV genome as a *cis*-acting element for the initiation of replication has been suggested when *E. coli*-expressed RdRp bound to the 3' UTR and initiated RNA synthesis. The two stem-loop (SL) structures, SL1 and SL2, at the 3' end UTR and the poly (A) tract were proposed to be critical for RdRp binding during HEV replication (3).

The ORF2 of HEV encodes the major capsid protein (pORF2) of 660 amino acids, required for virion production and infectivity (47). The interaction of the ORF2 protein with the 5' end of the HEV genome might play a role in the encapsidation of HEV RNA (84). Three potential N-linked glycosylation sites were identified in ORF2 protein, and mutations that eliminated glycosylation sites resulted in loss of infectivity in rhesus macaques and the formation of non-infectious viral particles. However, the biological relevance of glycosylation in non-enveloped HEV is not known (49). The Recombinant ORF2 protein expressed in insect cells is truncated at its N-terminus and C-terminus. The 56 kDa protein (aa 112-607) encoded by ORF2 expressed in insect cells can assemble into virus-like particles (VLPs) (81). The C-terminal region of the ORF2 protein is immunogenic, induces neutralizing antibodies, and includes a neutralization site mapped to the peptide region spanning the amino acids 458 to 607 (159). The crystal structure of the truncated ORF2 protein has been reported (50, 151). The HEV capsid protruding domain is dimeric and dimerization is essential for HEV-host interaction (78). The protruding domain might also play a role in antigenicity and virus neutralization (50, 78). Studies on hepatitis E virus-like particles (HEV-LP) assembled from truncated HEV capsid protein (aa 112 to 608), revealed the importance of aromatic amino acids at the 5-fold axis in particle

formation, as well as the presence of putative receptor-binding regions and neutralizing epitopes on the 3D structure of HEV-LP (151).

The ORF3 of HEV, which overlaps the N-terminus of ORF2, encodes a small protein of 123 amino acids and has a molecular mass of approximately 13 kDa (48, 59, 129). The ORF2 and ORF3 proteins are encoded by a bicistronic subgenomic RNA (48, 129, 149). It was demonstrated that the third methionine codon in ORF3 is used to initiate translation, and the translated protein is shorter by 9 amino acids at its N-terminus (48). Although, the ORF3 protein is dispensable for HEV replication *in vitro*, it is absolutely required for infection *in vivo* (33). The protein is phosphorylated at a single serine residue (Ser-80) and is associated with the cytoskeleton. The interaction of phosphorylated ORF3 with nonglycosylated ORF2 may have a role in virion assembly (135, 140). The proline-rich region of ORF3 binds to the src homology 3 domain of cellular proteins and has been postulated to be a viral regulatory protein that modulates cell signaling pathways (65, 74). Release of HEV from cells is dependent on ORF3 protein both *in vitro* and *in vivo* (34, 150). The ORF3 protein may play a role in cell survival through EPK activation (65) and protection of cells from mitochondrial depolarization (97), and modulation of the acute phase response that favors viral replication (27).

HEV replication

Based on similarities and sequence homology to that of other well characterized positive-strand RNA viruses, a model of HEV replication has been proposed (28, 60). After entry of the virus into the permissive cell, genomic RNA is uncoated. The ORF1 gene is translated into a nonstructural polyprotein, which is cleaved by proteases. A negative strand replicative intermediate is synthesized by viral-RdRp, which serves as template for the synthesis of positive-stranded subgenomic RNA and full-length genomic RNA (Figure 1.2). A region homologous to the junction region of alphaviruses serves as a subgenomic promoter (59). The subgenomic RNA synthesized is then translated into structural proteins during the later stages of replication. Once the viral genome is packaged into the capsid protein, the progeny virus exits the cell through an undefined pathway. Although, the proposed strategy is not experimentally demonstrated, several studies have supported this replication strategy. The requirement for a 5' end capped structure for HEV replication (38), is compatible with the cap-dependent translation of HEV genomic RNA. The presence of HEV genomic (~7.5 kb) and two subgenomic (~3.7 kb and ~2 kb) RNAs

in experimentally infected cynomolgus macaques has been demonstrated (130). Positive and negative strands of HEV RNA have been detected in the livers of experimentally infected rhesus monkeys (100) and pigs (91). It was recently shown that the viral-RdRP binds to the 3' end of HEV RNA to initiate RNA synthesis, and this polymerase activity is necessary for genome replication (3). The infectious nature of capped RNA transcripts from the cDNA clones indicates that subgenomic RNAs are not required for initiation of the infection.

HEV in animals

Swine hepatitis E virus (swine HEV)

The experimental infection of Russian swine with an Asian strain of human HEV was reported by Balyan et al. in 1990(13). However, the virus isolate in the study was not sequenced to confirm its identity. Swine HEV was the first animal strain of HEV to be identified and characterized from pigs in the US (94). Swine HEV shares about 79-80 % nucleotide sequence identity and 90-92 % amino acid sequence identity in the ORF2 region with human HEV. The ORF3 region of swine shares about 83-85 % nucleotide sequence identity and 77-82 % amino acid sequence identity (94). Since its initial identification in 1997, swine HEV has been identified in pigs worldwide, and the isolates identified thus far are genetically closely related to the genotypes 3 and 4 strains of human HEV. Phylogenetic analysis revealed that HEV strains from a given geographical region are of the same genotype (8, 9, 14, 15, 25, 30, 43, 104, 126). Genotypes 1 and 2 HEV strains are restricted to humans and have not as yet been definitively identified in swine.

Avian HEV

Payne et al., (1999) identified a novel virus associated with big liver and spleen disease (BLSD) in chickens from Australia (107). A short fragment of 532 bp of the big liver and spleen disease virus (BLSV) shared a 62% sequence identity with the human HEV . Hepatitis-Splenomegaly (HS) syndrome in chickens, which is related to BLSD was first reported in Canada (114). Hepatitis-Splenomegaly is a disease commonly found in 30-72 week-old broiler breeders and layers, associated with drop in egg production and increased mortality (114). Chickens with HS syndrome show a moderate to poor body condition. The gross lesions include swollen liver with hemorrhages, enlarged spleen and ovarian regression. The disease is typically

a more hemorrhagic disease than BLSD with clotted blood or blood stained fluid present in the abdomen of affected birds (114).

In 2001, Haqshenas et al. first isolated a virus associated with hepatitis-splenomegaly (HS) syndrome in the United States (53). Electron microscopy of bile samples from chickens with HS syndrome revealed non-enveloped viral particles of 30-35 nm in diameter, with similar size and morphology to those of human HEV (53). Based upon significant genetic relatedness with and similar genomic organization to mammalian HEV, the novel virus in chickens has been designated as avian HEV. Avian HEV shares a 80% nucleotide identity with the short fragment sequenced in BLSV (53, 107), and a 57-61 % nucleotide sequence identity with mammalian HEVs in the highly conserved helicase region (53). Phylogenetic analysis based on complete genomic sequence confirmed that avian HEV is a distinct branch segregated from genetically related human and swine HEVs of the four genotypes. Avian HEV displays a 50% nucleotide sequence identity over the complete genome, 48-51 % identity in ORF1, 46-48% in ORF2 and only 29-34 % identity in ORF3 with mammalian strains. The putative polyprotein encoded by ORF1 shares a 41-42 % identity with mammalian strains of HEV(57). The helicase gene is the most conserved region between avian HEV and mammalian HEV strains (53, 57). The capsid protein of avian HEV shares common antigenic epitopes with that of human and swine HEV strains (52). Thus far, at least three distinct genotypes of avian HEV have been identified from chickens worldwide (17, 53, 158). Significant similarities in structural and functional features with that of mammalian HEVs supported the inclusion of avian HEV in the family *Hepeviridae* (57), although avian HEV likely belongs to a separate genus.

Specific-pathogen-free (SPF) chickens experimentally infected with the prototype strain of avian HEV developed mild gross pathological lesions characteristic of HS syndrome. These included subscapular hemorrhages and enlargement of the liver, and microscopic liver lesions which includes lymphocytic phlebitis and periphlebitis with occasional biliary vacuolation, amorphous hypocellular eosinophilic matrixes, hemorrhages, and necrotic foci. Avian HEV infection in chickens, which is associated with a hepatic disease (HS syndrome), provides an homologous animal model system for the study of HEV pathogenesis and replication of HEV (18).

HEV strains from other animal species

Besides domestic pigs and chickens, HEV antibodies have also been detected in a wide range of animal species, including wild boar, cattle, sheep, goats, mongooses, horses, deer, rats, dogs, cats, and rabbits (62, 98, 111, 116, 134, 156, 157). Novel strains of HEV have only been genetically identified from chickens, wild boar, sika deer, mongoose, rats, and rabbits (53, 62, 157). The source of HEV seropositivity in other animal species remains unknown (88).

HEV in wild boar, deer, and mongoose

HEV infection in wild boar is reported in many countries including Germany, Italy, Spain, Australia, and Japan (93). In 2004, Sonoda et al, first isolated an HEV strain from a Japanese wild boar (123). The complete genomic sequence of HEV recovered from the wild boar showed a 99.7 % sequence identity with deer-derived HEV, and 99.9 % sequence identity with isolates from patients who developed hepatitis after consumption of deer meat (125). The report suggests interspecies transmission between boar and deer in their wild life in shared habitat, and the source of HEV infection for human beings who consume raw meat. The complete genome of HEV isolated from mongoose was sequenced and shown to be a genotype 3 strain that was closely related to a swine HEV strain of the same genotype from Japan(99). The lower prevalence of anti-HEV antibodies in mongoose (8.3%) compared to pigs (58%), and wild boars (44%), suggests that mongooses may not be the major reservoir of HEV, although human cases associated with this reservoir have not been reported (80).

HEV in rats

The seroprevalance of HEV antibodies varies among rodent populations of different geographical regions; this includes wild rats (*Rattus norvegicus*, *Rattus rattus* and *Rattus exulans*), and other rodent species. The serological findings were confirmed by the genetic identification of a strain of HEV in the feces of wild rats in Germany (62). A short fragment of 4019 bp of rat HEV was shown to be closely related to genotypes 1 and 3 of human HEV strains, but with only 58.4 % nucleotide sequence identity. The deduced amino sequence identities ranged from 52.1 to 58.7 % for the partial ORF1, 42.9 to 56.2 % for ORF2 and 24.8 to 32.8 % for ORF3 with those of genotypes 1 to 4 avian HEV. Phylogenetic analysis based on the deduced amino acid sequence placed rat HEV as a distinct group from the remaining two groups, which

includes avian HEV in one group, and the remaining HEVs isolated from human and different animal species in the third group (62). The ability of rat HEV to cross species barrier remains unknown.

HEV in rabbits

A novel strain of HEV, rabbit HEV, was recently identified from farmed rabbits in China (157). Approximately 57 % of the farm rabbits were positive for anti-HEV antibodies, and 7.6 % for HEV RNA. Rabbit HEV was suggested to be a novel genotype genetically related to mammalian HEVs, and closely related to genotype 3. The full-length genomic sequences were shown to have 74 %, 73 %, 78 -79 %, 74-75 %, and 46-47 % identity to genotypes 1, 2, 3, 4, and avian HEV, respectively. Rabbits that were experimentally inoculated with rabbit HEV showed the typical markers of liver infection, i.e., antibodies appeared after the detection of viral antigen. Experimental challenge of rabbits with high doses of virus in terms of genome equivalents resulted in elevated serum ALT levels. In turn this suggested an acute infection, and local hepatocellular necrosis in liver sections, indicating the possible suitability of rabbits as animal model for HEV infection (82). In contrast to the analysis by Zhao et al., (157), where rabbit HEV was classified as a novel genotype, rabbit HEV was shown in another study to be closely related to genotype 3 strains and placed in the same group as other mammalian HEVs (53). It is not known if rabbit HEV can cross the species barrier and infect humans and other animal species. However, it has been shown that HEV genotype 4 crosses the species barrier to infect rabbits, but with lower infectivity and pathogenicity than seen in non-human primate models (82).

Animal reservoirs, Cross-species infection, and Zoonotic importance of HEV

Prevalence of HEV antibodies in pigs in developing countries as well as industrialized countries suggests that HEV is enzootic in pigs worldwide (88, 90). Seroprevalence of HEV has also been reported in other animal species, including cattle, sheep, goats, horses, non-human primates, dogs, and cats, suggesting the exposure of these animal species to HEV or a HEV-like agent (88). Since the discovery of the first animal strain of HEV, swine HEV, many HEV strains have been identified in swine population across the world. HEV-like viruses were identified from chickens with Hepatitis-Splenomegaly (HS) syndrome in North America, farmed rabbits in

China, and wild rats in Germany (53, 62, 157). HEV strains were also genetically identified from wild boar, sika deer, and mongoose in Japan (99, 123, 125). The close genetic relationship between swine HEV strains and human HEV strains of genotypes 3 and 4 in their respective geographical regions, suggests pigs as possible natural reservoir hosts for genotype 3 and 4 strains of HEV.

Interspecies transmission of genotype 3 swine HEV to rhesus monkeys and a chimpanzee, and US-2 strain of human HEV to SPF pigs, was demonstrated experimentally (51, 92). Similarly, it was shown that genotype 4 human HEV can also cross species barrier to infect SPF pigs under experimental conditions (40). However, experimental infection of SPF pigs with a genotype 1 human HEV strain (Sar-55) and the genotype 2 human HEV strain (Mex-14) were unsuccessful (91), suggesting that the genotypes 1 and 2 of human HEV have limited host range and are confined to humans. Further, there is an experimental report that genotype 4 human HEV have the ability cross species and infect rabbits, whereas genotype 1 human HEV failed to infect rabbits (82). The interspecies transmission studies involving genotype 3 and/or genotype 4 HEV suggest possible broader host specificity of these genotypes. Besides mammalian HEV, cross-species infection of turkeys with avian HEV was reported (124). However, an attempt to experimentally infect rhesus monkeys with avian HEV was unsuccessful, suggesting that avian HEV may not infect humans (57).

Sporadic cases of zoonotic transmission of HEV have recently been documented in individuals who consumed raw or undercooked pig livers in Japan (153). The first direct evidence of HEV infection as a zoonosis was provided when HEV infection was detected among people who consumed raw deer meat, and the human isolate was found to be identical to HEV detected in frozen meat of same deer (133). Swine veterinarians and pig handlers were reported to be at increased risk of HEV zoonosis, both in developing and industrialized countries (89). The presence of HEV antibodies across different species of animals, genetic identification of new animal strains of HEV, seroprevalance of HEV in swine veterinarians and swine handlers, and food-borne transmission of HEV by the consumption of raw or undercooked meat and meats products, suggests the existence of animal reservoirs for HEV and its zoonotic potential.

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Figure 1.1

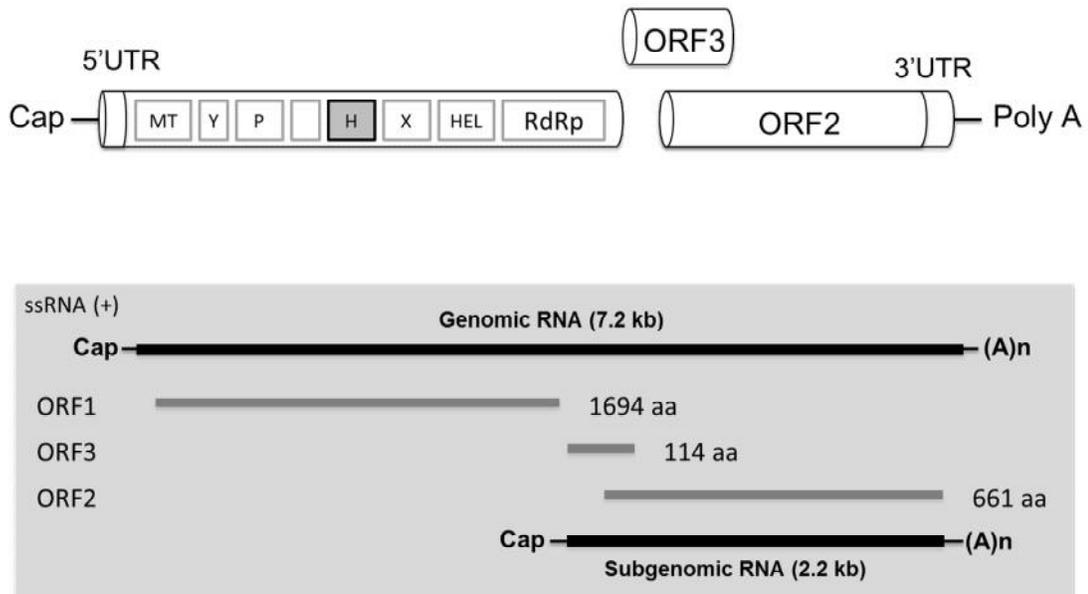


Figure 1.1. Schematic diagrams of the organization of HEV genome. The putative functional domains: MT, methyltransferase; Y, Y-domain; P, papain-like cysteine protease; HEL, helicase; and RdRp, RNA-dependent RNA polymerase are marked by boxes, while the H, HVR (aa707-775) is boxed in grey.

Figure 1.2

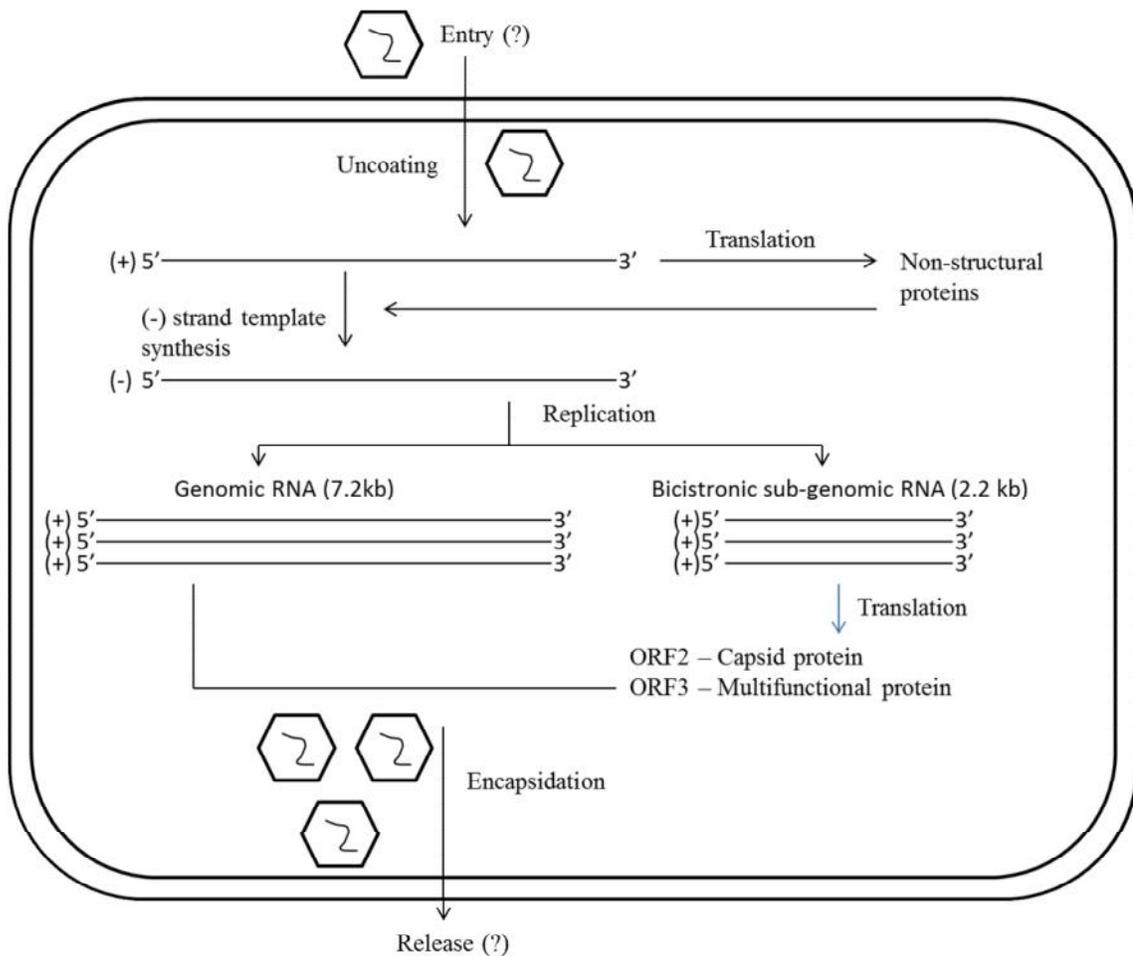


Figure 1.2. Schematic representation of a model of HEV replication strategy. The virus enters the cell and the genome is uncoated. The genome is translated by ribosomes to produce non-structural polyprotein, which is cleaved by protease. The viral genome is therefore messenger sense and also functions as a template for the synthesis of negative-strand RNA. The RdRp uses the negative strand (or anti-genome) as a catalytic template for the production of new genomic RNA and bicistronic sub-genomic RNA. The subgenomic RNA synthesized is then translated into structural proteins during the later stages of replication. Once the viral genome is encapsidated, the progeny virus exits the cell through an undefined pathway.

Chapter 2

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*

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This chapter was published by *Journal of Virology*, Jan 2009; 83(1):384-395, doi:10.1128/JVI.01854-08 and is used with kind permission of American Society for Microbiology (ASM).

ABSTRACT

Hepatitis E virus (HEV) is an important human pathogen, although little is known about its biology and replication. Comparative sequence analysis revealed a hypervariable region (HVR) with extensive sequence variations in open reading frame 1 of HEV. To elucidate the role of the HVR in HEV replication, we first constructed two HVR deletion mutants, hHVRd1 and hHVRd2, with in-frame deletion of amino acids (aa) 711 to 777 and 747 to 761 in the HVR of a genotype 1 human HEV replicon. Evidence of HEV replication was detected in Huh7 cells transfected with RNA transcripts from mutant hHVRd2, as evidenced by expression of enhanced green fluorescent protein. To confirm the *in vitro* results, we constructed three avian HEV mutants with various HVR deletions: mutants aHVRd1, with deletion of aa 557 to 585 (Δ 557-585); aHVRd2 (Δ 612-641); and aHVRd3 (Δ 557-641). Chickens intrahepatically inoculated with capped RNA transcripts from mutants aHVRd1 and aHVRd2 developed active viral infection, as evidenced by seroconversion, viremia, and fecal virus shedding, although mutant aHVRd3, with complete HVR deletion, was apparently attenuated in chickens. To further verify the results, we constructed four additional HVR deletion mutants using the genotype 3 swine HEV as the backbone. Mutants sHVRd2 (Δ 722-781), sHVRd3 (Δ 735-765), and sHVRd4 (Δ 712-765) were shown to tolerate deletions and were infectious in pigs intrahepatically inoculated with capped RNA transcripts from the mutants, whereas mutant sHVRd1 (Δ 712-790), with a nearly complete HVR deletion, exhibited an attenuation phenotype in infected pigs. The data from these studies indicate that deletions in HVR do not abolish HEV infectivity *in vitro* or *in vivo*, although

evidence for attenuation was observed for HEV mutants with a larger or nearly complete HVR deletion.

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of human hepatitis E, is a nonenveloped, single-stranded, positive-sense RNA virus in the genus *Hepevirus* of the family *Hepeviridae* (9). Hepatitis E is an important public health disease in many developing countries and is also endemic in some industrialized countries (1, 2, 4, 8, 19, 41). HEV transmission occurs primarily by the fecal-oral route through contaminated drinking water or water supplies in areas with poor sanitation (35). The disease mainly affects young adults, and the mortality rate is generally less than 1%, but it can reach up to 28% among pregnant women (17, 28). A relatively high prevalence of HEV antibodies in healthy individuals has been reported in the United States (US) and other industrialized countries where HEV infections are only sporadic (33, 34, 51). HEV antibodies have also been detected in several other animal species, including rodents, pigs, and chickens (12, 30, 35, 54). In 1997, the first animal strain of HEV, swine HEV, was discovered and characterized from pigs in the United States (38). More recently, another strain of HEV, avian HEV, from chickens with hepatitis-splenomegaly syndrome was discovered and characterized in the US (18). The discovery of animal strains of HEV and the existence of a population of individuals in industrialized countries who are seropositive for HEV have led to a hypothesis that animal reservoirs exist for HEV (34). Increasing evidence indicates that hepatitis E is indeed a zoonotic disease (35, 39) and that pigs (and perhaps other species) are animal reservoirs for HEV (34).

There are at least four major genotypes of HEV: genotype 1 (primarily Burmese-like Asian strains) (3, 50, 52); genotype 2 (a single Mexican strain) (21); genotype 3 (strains from rare endemic cases in industrialized countries, including the US, Europe (Eu), and Japan, and swine HEV strains from pigs worldwide) (11, 38, 45-48); and genotype 4 (variant strains from endemic cases in Asia and swine HEV strains from pigs in Asia) (49, 57). All swine HEV strains identified thus far worldwide belong to either genotype 3 or 4 (20, 22, 40, 47). The avian HEV from chickens likely represents a new genus in the family *Hepeviridae* (25).

The genome of HEV is approximately 7.2 kb in length and consists of three open reading frames (ORFs) (14, 31, 43, 50, 55) and short 5' and 3' noncoding regions (NCR), followed by a

poly(A) tail (8, 15, 29, 50). The ORF1 encodes a nonstructural polyprotein, which contains putative functional motifs characteristic of methyltransferase, protease, helicase, and RNA-dependent RNA polymerase (31, 42). ORF2 encodes the capsid protein of about 660 amino acids (aa). The small ORF3 encodes a phosphoprotein of about 123 aa, the biological function of which has yet to be fully defined (14, 15, 31, 50, 55, 58, 60). Though ORF1 is essential for HEV replication, it contains a highly heterogeneous and hypervariable region (HVR) among HEV strains (25, 32, 44, 53). The size differences among different HEV genomes are confined mainly to the HVR of ORF1 (25). The observed extensive inter- and intragenotypic sequence variations in the HVRs of HEV genomes suggest that the HVR may not be necessary for virus replication. However, sequences not required for virus infectivity or spread are normally rapidly lost *in vivo*, especially in small RNA viruses like HEV. Therefore, the fact that HEV does retain such a hypervariable sequence in its genome suggested a potential biological role for the HVR in HEV replication and/or pathogenesis, which warranted further investigation.

Reverse genetic systems for HEV have been recently established, permitting the manipulation of the HEV genome to explore the potential functions of viral genes (10, 24, 26). To elucidate the potential role of the HVR in HEV replication and/or pathogenesis, in this study we constructed various HVR deletion mutants using a genotype 1 human HEV replicon, an avian HEV infectious clone, and genotype 3 swine HEV infectious clones. The mutants were tested for infectivity in Huh7 liver cells, as well as in chickens and pigs. The results from this study indicate that deletions of HVR from the HEV genome do not affect virus viability *in vitro* or *in vivo*, although virus mutants with a larger or nearly complete HVR deletion were apparently attenuated in infected animals.

MATERIALS AND METHODS

Cells and infectious cDNA clones. The genotype 1 human HEV (Sar55 strain) infectious clone (10) and a subclone of the Huh7 liver cell line (7, 15) were gifts from Suzanne Emerson and Robert Purcell at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH. The infectious cDNA clones of the genotype 3 swine HEV (26) and avian HEV (24) were reported previously.

Sequence analysis of the HVR among known HEV strains. In order to identify the length of the HVR and to determine the intragenotypic and intergenotypic sequence identities in the HVR

among different HEV strains of the four major genotypes, the amino acid sequences flanking the HVR among known mammalian strains of HEV (Figure 2.1) and the corresponding region of avian HEV were aligned and analyzed using the Clustal W method of the MegAlign program (DNASar, Inc.). The putative HVR for each genotype of mammalian HEV and the corresponding HVR in avian HEV were predicted on the basis of the sequence alignment.

Construction of genotype 1 human HEV HVR deletion mutant replicons. The enhanced green fluorescent protein (EGFP)-expressing HEV replicon was constructed in our laboratory (Y. W. Huang and X. J. Meng, unpublished data) using the infectious cDNA clone of genotype 1 human HEV (strain Sar55), pSK-HEV-2 (10), as the backbone. Briefly, the 5' NCR, ORF1, and 3' NCR in this EGFP replicon are intact, with part of the carboxy terminus of ORF2 fused to the EGFP gene. The amino terminus of the ORF2 gene (nt 5148 to 5816) downstream of the first methionine was removed, and the EGFP gene was inserted in frame with the ORF2 initiation codon. The EGFP replicon was shown to express the EGFP protein when transfected into Huh7 liver cells (Huang and Meng, unpublished data).

Two HVR deletion mutants of the genotype 1 HEV replicon were constructed using fusion PCR (Figure 2.2). Amino acid residues 711 to 777 and 747 to 761, corresponding to nucleotides (nt) 2131 to 2331 and 2239 to 2283, were deleted to construct the HVR deletion mutants hHVRd1 and hHVR2, respectively. The two fragments used for fusion PCR were first amplified with the primer sets Hu F/Hu r1 and Hu f1/Hu R for the mutant hHVRd1 and Hu F/Hu r2 and Hu f2/Hu R for the mutant hHVRd2 (Table 2.1). The PCR products amplified from each mutant were then used in the fusion PCR with primer set Hu F/Hu R (Table 2.1). To produce the two HVR deletion mutants, the fusion product was purified, digested with *SphI* and *NsiI*, and ligated into the backbone of the genotype 1 HEV EGFP replicon from which the *SphI*-*NsiI* region had been deleted.

Construction of avian HEV HVR deletion mutants using the avian HEV infectious cDNA clone as the backbone. To further elucidate the role of the HVR in HEV replication, we utilized the genetically distinct avian HEV to produce three avian HEV HVR deletion mutants. Avian HEV shares only approximately 50% nucleotide sequence identity with the mammalian HEV strains (23-25), although avian HEV and mammalian HEV have similar genomic organizations. Since the complete sequences of avian HEV are available for only two strains, the putative HVR in avian HEV is derived from the corresponding HVR in mammalian HEV.

To construct the three avian HEV HVR deletion mutants with various lengths, the avian infectious cDNA clone (24), pT7-aHEV, was used as the backbone (Figure 2.3). Mutant aHVRd1 was created by PCR to delete amino acid residues 557 to 585 (nt 1693 to 1779) using the primers Avf and AvR (Table 2.1). Similarly, to construct mutant aHVRd2, primers AvF and Avr were used to delete amino acid residues 612 to 641 (nt 1858 to 1947). All four primers contain a unique *HpaI* restriction site. The PCR products were purified with a GeneClean II kit, digested with *HpaI*, and ligated into the backbone of the avian HEV infectious clone pT7-aHEV from which the *HpaI* region had been deleted. For the construction of mutant aHVRd3, aa 557 to 641 (nt 1693 to 1947) were deleted by direct digestion of the avian HEV infectious cDNA clone with *HpaI* and religation of the ends after purification.

Construction of swine HEV HVR deletion mutants using the genotype 3 swine HEV infectious cDNA clone as the backbone. To more definitively verify the role of the HVR in HEV replication, we subsequently constructed four additional HVR deletion mutants with a different genotype, the genotype 3 swine HEV. Briefly, the infectious cDNA clone pSHEV-3 of the prototype genotype 3 swine HEV (26) was used as the backbone for the construction of four HVR deletion mutants using fusion PCR. Amino acid residues 712 to 790, 722 to 781, 735 to 765, and 712 to 765 (corresponding to nt 2160 to 2396, 2190 to 2369, 2229 to 2321, and 2160 to 2321, respectively) were deleted from the infectious cDNA clone pSHEV-3 to produce HVR deletion mutants sHVRd1, sHVRd2, sHVRd3, and sHVRd4, respectively (Figure 2.4). The two fragments used for fusion PCR were first amplified with the primer sets Sw F/Sw r1 and Sw f1/Sw R for the mutant sHVRd1, Sw F/Sw r2 and Sw f2/Sw R for the mutant sHVRd2, Sw F/Sw r3 and Sw f3/Sw R for the mutant sHVRd3, and Sw F/Sw r1 and Sw f4/Sw R for the mutant sHVRd4 (Table 2.1). PCR products amplified from each mutant were then used in the fusion PCR with primer set Sw F/Sw R (Table 2.1). The fusion PCR products were purified, digested with *XhoI* and *SexAI*, and ligated into the backbone of the genotype 3 swine HEV infectious cDNA clone pSHEV-3 from which the *XhoI-SexAI* region had been deleted.

In vitro transcription. For demonstration of the viability of the HVR deletion mutants in Huh7 liver cells, the genotype 1 human HEV EGFP replicon and its derived HVR deletion mutants (hHVRd1 and hHVRd2) were first linearized with *BglII* and purified by phenol-chloroform extraction and ethanol precipitation. Capped RNA transcripts were synthesized with the mMMESSAGE mMACHINE T7 kit (Ambion) from the mutant and wild-type replicons (24).

Briefly, each transcription reaction was performed in a 20- μ l reaction mixture containing 1 μ g linearized cDNA template, 2 μ l 10X reaction buffer, 10 μ l 2X nucleoside triphosphate/Cap, 2 μ l enzyme mixture, and an additional 1 μ l 30 mM GTP stock for capping. The mixtures were incubated at 37°C for 1.5 h, and 0.5 μ l of each reaction mixture was run on a 0.8% agarose gel to check the quality of the RNA transcripts. Each transcription mixture was cooled on ice and used for the transfection of Huh7 liver cells as previously described (7).

For avian HEV mutants, the full-length cDNA clone of avian HEV and the three avian HEV HVR deletion mutants were linearized by digestion with *XhoI* and purified by phenol-chloroform extraction. Each *in vitro* transcription reaction was performed in a 300- μ l reaction as described previously (24) to generate capped full-length RNA transcripts. RNA transcripts from each cDNA clone were diluted 1:4 with cold RNase-, DNase-, and proteinase-free phosphate-buffered saline (PBS) buffer, frozen on dry ice, and used for intrahepatic inoculation of chickens on the same day.

For the genotype 3 swine HEV mutants, the full-length cDNA clone of swine HEV and the four HVR deletion mutants were linearized with *XbaI* and purified. Capped RNA transcripts were synthesized from each cDNA clone in a 600- μ l reaction as described previously (27). Once the RNA transcripts were examined for quality on an agarose gel, the transcription reaction mixture from each clone was diluted with 4 volumes of cold RNase-, DNase-, and proteinase-free PBS buffer; aliquoted into 1-ml vials; and immediately frozen on dry ice until it was used for intrahepatic inoculation of pigs the next day.

In vitro transfection of Huh7 liver cells with genotype 1 human HEV HVR deletion mutant replicons. The Huh7 cells grown in a six-well plate were washed once with serum-free medium prior to transfection. Five microliters of the capped RNA transcripts generated from the wild type or HVR deletion mutants of the genotype 1 HEV EGFP replicon were mixed with 1 ml of Opti-MEM (Invitrogen) containing 10 μ l of DMRIE-C (Invitrogen), and the mixture was overlaid on cells in a drained well. After 4 h of incubation at 34.5°C, the mixture was aspirated, fresh Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was added, and the cultures were continuously incubated at 34.5°C. Expression of EGFP in transfected cells was examined on days 4, 5, and 6 post-transfection with a fluorescence microscope (Nikon).

Evaluation of the infectivity of avian HEV HVR deletion mutants in specific-pathogen-free (SPF) chickens. The lack of an efficient cell culture system for HEV propagation limited our

ability to test the viability and infectivity of mutant or wild-type viruses in cell cultures. We had previously developed a unique procedure (intrahepatic inoculation of chickens with capped RNA transcripts from avian HEV infectious clones via percutaneous injection) to successfully determine the infectivity of avian HEV cDNA clones (24). Therefore, to test the infectivity and replication competency of the avian HEV HVR deletion mutants, we utilized the percutaneous-injection procedure for intrahepatic inoculation of chickens with capped RNA transcripts from the wild-type avian HEV infectious clone and HVR deletion mutants as described previously (24).

Briefly, 15 11-week-old SPF chickens that were negative for avian HEV RNA and antibodies were divided into five groups (groups A through E), with 3 chickens in each group (Figure 2.3). The RNA transcripts were injected immediately, after a quick thaw, into four different sites in each liver, with approximately 100 μ l per injection site. Three chickens in group A (5357, 5361, and 5368) were each injected with 400 μ l of RNA transcripts from mutant aHVRd1; chickens 5353, 5372, and 5388 in group B with RNA transcripts from mutant clone aHVRd2; and chickens 5354, 5355, and 5385 in group C with RNA transcripts from mutant aHVRd3. Chickens 5362, 5367, and 5374 in group D were each injected with RNA transcripts from the wild-type avian HEV infectious clone and served as positive controls. The three chickens in group E (5352, 5358, and 5369) were injected similarly with PBS buffer as negative controls (Figure 2.3). Fecal swabs and sera were collected from each inoculated chicken at weekly intervals and tested by RT-PCR for avian HEV RNA. Weekly serum samples were also tested by enzyme-linked immunosorbent assay (ELISA) for seroconversion to avian HEV antibodies as previously described (23, 25). All inoculated chickens were necropsied at 8 weeks postinoculation (p.i.).

Determination of the infectivities of the genotype 3 swine HEV HVR deletion mutants in SPF pigs. The *in vivo* transfection system developed in our previous studies for testing the infectivities of swine HEV infectious cDNA clones and mutants (26) was used to determine the infectivities of HVR deletion mutants in pigs. Briefly, 18 6-week-old SPF pigs that were seronegative for HEV were assigned to six groups of three each (groups A, B, C, D, E, and F). An ultrasound-guided technique was used to inoculate the pigs intrahepatically with capped RNA transcripts from each of the mutants, as described previously (26). The RNA transcripts were thawed and immediately injected into five different sites in each liver with 200 μ l per

injection site. The three pigs (no. 286, 296, and 608) in group A were each injected with 1 ml of the RNA transcripts from mutant sHVRd1. Similarly, pigs 291, 295, and 613 in group B were each injected with RNA transcripts from mutant sHVRd2; pigs 292, 604, and 606 (group C) with RNA transcripts from sHVRd3; and pigs 288, 293, and 300 (group D) with the RNA transcripts from mutant sHVRd4. The three pigs 603, 609, and 611 in group F were each injected similarly with 1 ml of PBS buffer and served as negative controls, and the three pigs 289, 294, and 612 in group E were intrahepatically injected with 1 ml of the RNA transcripts from the wild-type pSHEV-3 infectious clone and served as positive controls (Figure 2.4). Fecal samples and sera were collected from all inoculated pigs at weekly intervals until they were necropsied at 10 weeks p.i. Fecal and serum samples were tested by reverse transcription (RT)-PCR (16, 36, 37, 59) for swine HEV RNA, and weekly serum samples were also tested by ELISA for immunoglobulin G (IgG) antibodies to swine HEV (16, 36).

Detection and sequencing of viruses recovered from experimentally infected chickens and pigs. For the chicken study, fecal materials collected from inoculated chickens at 3 weeks p.i. were tested by RT-PCR using the primers specific for the avian HEV HVR. A nested PCR with external primers Av N1 and Av N2 and internal primers Av N3 and Av N4 (Table 1) were used to amplify the region flanking the avian HEV HVR. Similarly, for the pig study, fecal materials collected from the inoculated pigs at 4 weeks p.i. were tested by RT-PCR using the primers specific for the genotype 3 swine HEV HVR. A One-step RT-PCR kit (Invitrogen) was used to amplify the region flanking the swine HEV HVR using the Sw N1 and Sw N2 primers (Table 2.1). The amplified PCR products from pigs and chickens were purified with a GeneClean II kit and sequenced at the Virginia Bioinformatics Institute (Virginia Tech). The sequences obtained from viruses recovered from the infected chickens and pigs were compared with the sequences of the original viruses used as the inocula.

RESULTS

The HVR is highly variable among HEV strains. Sequence analyses confirmed the existence of an HVR in ORF1 of HEV strains (Figure 2.1). The intergenotypic amino acid sequence identity in the HVR among HEV isolates in different genotypes differed by as much as 71%, whereas the intragenotypic amino acid sequence identities among isolates within the same

genotype differed by 31% among genotype 1 isolates, 41% among genotype 3 isolates, 46% among genotype 4 isolates, and 30% between the only two available avian HEV isolates (data not shown). The variability of the HVR in genotype 2 is unknown, since only one strain of genotype 2 HEV has been sequenced to date. The predicted HVR for the HEV strains used in the present study includes ORF1 aa 707 to 777 in genotype 1 human HEV (Sar55 strain) (Figure 2.2), aa 707 to 790 in genotype 3 swine HEV (pSHEV-3 infectious clone) (Figure 2.4), and aa 557 to 641 in avian HEV (Figure 2.3). It was previously predicted, based on sequence comparison of an apparently avirulent strain and the prototype pathogenic strain of avian HEV, that the region spanning aa 554 to 614 in ORF1 of avian HEV is hypervariable (5). However, further sequence comparisons with mammalian HEV strains revealed that the avian HEV genome downstream of the originally predicted HVR at aa 615 to 641 also displayed significant sequence variations. Therefore, we considered aa 557 to 641 the HVR of avian HEV for the purpose of constructing avian HEV HVR deletion mutants in this study (Figure 2.3).

The HVR of genotype 1 human HEV is not required for virus replication in vitro. We constructed two genotype 1 human HEV HVR deletion mutants using a strain Sar55 HEV replicon expressing EGFP (Figure 2.2). Huh7 cells were transfected with capped RNA transcripts from a wild-type replicon and the two mutants, hHVRd1 and hHVRd2. The transfected cells were examined by fluorescence microscopy on days 4, 5, and 6 posttransfection for evidence of EGFP expression. EGFP fluorescence signal was detected in Huh7 cells transfected with the wild-type Sar55 replicon, as well as in those transfected with the mutant hHVRd2 replicon (Figure 2.2), but not in the cells transfected with the mutant hHVRd1 replicon. Fluorescence was first detected on day 4, and the EGFP signal intensity increased on days 5 and 6 posttransfection. Expression of EGFP by the HVR deletion mutant hHVRd2 indicated that the mutant is replication competent in Huh7 liver cells.

The HVR (aa 557 to 585 and aa 612 to 641) of avian HEV tolerated deletions. To validate the dispensability of the HVR for HEV replication observed in our *in vitro* study with the genotype 1 human HEV replicon, we selected the genetically distinct avian HEV for an *in vivo* study. Three avian HEV HVR deletion mutants were generated using the avian HEV infectious cDNA clone as the backbone (Figure 2.3). The abilities of the three avian HEV HVR deletion mutants to infect chickens were tested by direct intrahepatic inoculation of SPF chickens with capped RNA transcripts from each mutant. Seroconversion to IgG anti-avian HEV was observed

in all HVR deletion mutant groups (A, B, and C), as well as in the positive control group (D). In each group, however, only one or two chickens out of the three that were inoculated seroconverted (Figure 2.3): only one chicken (no. 5361) in group A (aHVRd1) seroconverted at 6 weeks p.i., chickens 5388 and 5353 of group B (aHVRd2) seroconverted at 4 and 6 weeks p.i., and chicken 5354 of group C (aHVRd3) seroconverted at 5 weeks p.i. All the chickens in the positive control group D seroconverted at 4 weeks p.i. The three negative control chickens (5352, 5358, and 5369) remained seronegative through the experiment.

Avian HEV-specific RNA in feces was detected variably in inoculated chickens (Table 2.2). In group A chickens (aHVRd1), fecal virus shedding began at 3 weeks p.i. for chicken 5361. Fecal virus shedding was delayed until 6 weeks p.i. in chicken 5357 and was not detected in chicken 5368. In group B chickens (aHVRd2), fecal virus shedding began at 2 and 3 weeks p.i. for chickens 5388 and 5353 but was undetectable in chicken 5372. None of the chickens in group C (aHVRd3) had detectable avian HEV RNA in the feces. Viremia could not be detected in group A or C chickens (Table 2.2) and was transient in chicken 5353 but lasted for 4 weeks in chicken 5388 of group B. Transient viremia was detected in all chickens of positive control group D.

The HVR of genotype 3 swine HEV is not required for *in vivo* infectivity. To further verify the results from the avian HEV chicken study and the *in vitro* genotype 1 HEV replicon study, we subsequently constructed four swine HEV mutants using the genotype 3 swine HEV infectious cDNA clone as the backbone (Figure 2.4). The abilities of the four genotype 3 swine HEV mutants to infect pigs were tested by direct intrahepatic inoculation of SPF pigs with capped RNA transcripts from each mutant. Since the intrahepatic inoculation of RNA transcripts of avian HEV HVR mutants in chickens was a blind percutaneous procedure (24), some chickens may not have received, or received much less, inocula in the livers. To ensure that all animals received equal amounts of RNA inocula, in the pig study with the genotype 3 HEV HVR deletion mutants, we used an ultrasound-guided technique for the intrahepatic injection to make sure that the RNA inocula were injected directly into the liver. All the pigs in groups A, B, C, and D, which were injected with capped RNA transcripts from respective HVR deletion mutants, seroconverted to IgG anti-HEV, indicating that active swine HEV infections had occurred in the inoculated pigs (Figure 2.4). All the pigs in group B (sHVRd 2), group C (sHVRd 3), and group D (sHVRd 4) seroconverted at about 3 to 5 weeks p.i. The three pigs in group A (sHVRd 1) had

a delayed seroconversion at 6 to 7 weeks p.i. The positive control pigs in group E (pSHEV-3) seroconverted at 3 to 4 weeks p.i. The three negative control pigs in group F remained seronegative throughout the course of study (Figure 2.4).

Fecal virus shedding occurred variably in pigs of groups B, C, and D (Table 2.3). There was no fecal virus shedding in group A pigs. Delayed fecal virus shedding occurred in pigs 291 and 295 of group B at 9 to 10 weeks p.i., while there was no fecal virus shedding in pig 613. Fecal virus shedding occurred as early as 1 week p.i. in group C pigs and at 2 weeks p.i. in group D pigs and lasted for 5 to 8 weeks. Viremia was not detected in group A or B pigs (Table 2.3). Viremia was detected only in pig 604 of group C at 3 and 6 weeks p.i. and only in pig 288 of group D at 5 weeks p.i. (Table 2.3).

Viruses recovered from infected chickens and pigs retained their respective deletions in the HVR. Viruses recovered from the feces of chicken no. 5361 from group A and chicken no. 5388 from group B at 3 weeks p.i. were sequenced to confirm the presence of deletions in the HVR. Sequence analyses revealed that the recovered virus from chicken no. 5361 retained its nt 1693 to 1779 deletion and the virus recovered from chicken no. 5388 also retained its nt 1858 to 1947 deletion. Similarly, we also amplified and sequenced the HVRs of the rescued viruses from selected pigs inoculated with the genotype 3 HVR deletion mutants. The introduced deletions nt 2190 to 2369 for group B pigs, nt 2229 to 2321 for group C pigs, and nt 2160 to 2321 for group D pigs were retained intact in the viruses recovered from the fecal samples collected at 4 weeks p.i.

DISCUSSION

The objective of this study was to assess the role of the HVR in ORF1 of HEV in virus replication and/or pathogenesis. Sequence analysis of known HEV strains revealed an HVR with a high degree of variability at both amino acid and nucleotide sequence levels. This region overlaps the proline-rich hinge region of ORF1 (25, 32, 44, 53). It is known that inherent structural constraints can influence the vulnerability of genomic segments to replication errors during virus infection, resulting in the accumulation of mutations for genetic diversity (13). The size differences in HEV genomes from different genotypes are confined mainly to the HVR of ORF1, which spanned 105 aa as originally proposed (31, 53). As the sequences of additional

HEV isolates were published, it became clear that the first 35 aa in the originally described HVR among HEV strains (53) is not hypervariable. Thus, the true HVR is 70 to 72 aa for genotype 1 HEV, 68 aa for genotype 2 HEV, 80 to 86 aa for genotype 3 HEV, 84 aa for all genotype 4 HEVs, and 84 aa for avian HEV (based on the corresponding region in mammalian HEVs). HEV genomes exhibited increased divergence in the HVR encompassing aa 707 to 777 for genotype 1 human HEV, aa 707 to 790 for genotype 3 swine HEV, and aa 557 to 641 for avian HEV. Extensive sequence variations observed among isolates in the four major genotypes of mammalian HEV and avian HEV, as well as within each genotype, suggested that the HVR may not be necessary for virus replication. It has been shown that a 507-nt deletion in a variable nonstructural region of rubella virus, a virus distantly related to HEV, is not required for virus replication (56). Therefore, we hypothesize that the HVR of HEV is not required for virus infectivity.

To test our hypothesis, we first constructed two genotype 1 human HEV HVR deletion mutants using the EGFP-expressing Sar55 HEV replicon as the backbone: mutants hHVRd1 (aa 711 to 777 deleted) and hHVRd2 (aa 747 to 761 deleted). The wild-type Sar55 EGFP replicon, which was constructed in our laboratory (Huang and Meng, unpublished), was shown to be replication competent and expressed EGFP when transfected into Huh7 liver cells. The two HVR deletion mutants (hHVRd1 and hHVRd2) were tested for viability and replication competency in Huh7 cells. EGFP fluorescence signal was detected in Huh7 cells transfected with the mutant hHVRd2, as well as with the wild-type Sar55 replicon, but not in the cells transfected with the HVR deletion mutant hHVRd1. The results from this experiment showed that the mutant hHVRd2 with partial HVR deletion is viable, and thus, the HVR is dispensable for virus replication *in vitro*. The absence of EGFP expression for mutant hHVRd1, which contains a deletion of the nearly complete HVR, suggested that this mutant is not replication competent. Therefore, it is likely that the end sequences of the HVR for genotype 1 human HEV may be important for virus viability. Nevertheless, partial deletion of HVR sequence in the middle region, as revealed by mutant hHVRd2 (Figure 2.2), apparently does not affect the replication ability of the genotype 1 human HEV *in vitro*.

To further confirm our results from the *in vitro* study with genotype 1 human HEV replicon mutants, we utilized a genetically distinct chicken strain of HEV (avian HEV); we constructed three avian HEV mutants with various deletions in the HVR and tested the mutants

for the ability to infect chickens. Based on the amino acid sequence alignment of avian HEV with other mammalian HEV strains, we found that the region spanning aa 557 to 641 in avian HEV is highly divergent and thus termed it the HVR for avian HEV. A total of three avian HEV HVR deletion mutants with various lengths were constructed: aHVRd1, with a partial deletion in the 5' end of the HVR; aHVRd2, with a partial deletion in the 3' end of the HVR; and aHVRd3, with the deletion of the nearly complete HVR (Figure 2.3). The infectivities of the three avian HEV mutants were tested in chickens by intrahepatically inoculating the RNA transcripts from each mutant into the livers of live chickens. The kinetics of virus replication appears to be different in chickens infected with different mutants and wild-type avian HEV (Figure 2.3). Although seroconversion was observed in chickens inoculated with all three mutants, only one or two out of the three inoculated chickens had seroconverted (Figure 2.3). Since the percutaneous intrahepatic-injection procedure used in this study to inoculate RNA transcripts into chicken livers is a blind procedure (24), it was quite possible that the RNA transcripts were not injected into the livers of some chickens or that only a small amount was injected (24). This may explain why not all inoculated chickens seroconverted to avian HEV antibodies. Fecal virus shedding and viremia were detected only in mutants aHVRd1 and aHVRd2. Deletions in the HVR may influence the replicative competence of the virus and thus may attenuate avian HEV. Therefore, attenuation of HVR deletion mutants to replicate at lower levels could explain why viral RNA was not detected in sera from group A chickens (aHVRd1) or in feces and sera of group C chickens (aHVRd3). Clearly, future studies are warranted to explore any potential role of the HVR in virus attenuation, which is beyond the scope of this study. The results from this avian HEV and chicken study indicated that the intact HVR of avian HEV is not essential for virus infectivity *in vivo*, although the avian HEV mutant with complete HVR deletion displayed an apparent attenuation phenotype.

In order to definitively verify our results from the avian HEV and chicken study, as well as from the *in vitro* genotype 1 HEV replicon mutant study, we subsequently constructed four genotype 3 swine HEV mutants with various HVR deletions: sHVRd1, with the deletion of the nearly complete HVR sequence; sHVRd2 and sHVRd3, with partial deletions of HVR sequences in the middle region; and sHVRd4, with a deletion of partial HVR sequence at the 5' end (Figure 2.4). The infectivities of these four mutants were tested in pigs by intrahepatic inoculation of capped RNA transcripts from each mutant via an ultrasound-guided inoculation procedure.

Similar to our observations in the chicken study, we found that mutants sHVRd2, sHVRd3, and sHVRd4, with partial deletions of the HVR sequences at the 5' end and in the middle region, are viable and infectious in pigs. Seroconversion was observed for all HVR deletion mutants; however, there was a delayed seroconversion with no detectable viral RNA in feces or sera for pigs inoculated with mutant sHVRd1, which has an nearly complete HVR deletion, an indication of attenuation for the sHVRd1 virus. Viral RNA was detected much later during infection, at 9 weeks p.i., in pigs (no. 291 and no. 295) infected with mutant sHVRd2, which contains a larger sequence deletion of the HVR than mutants sHVRd3 and sHVRd4. Fecal virus shedding was detected at 1 and 2 weeks p.i. in pigs inoculated with mutants sHVRd3 and sHVRd4 and lasted for 5 to 8 weeks. These results suggest that mutants sHVRd1 and sHVRd2, with larger sequence deletions of the HVR, may be attenuated to replicate at lower levels, and it appears that the lengths of HVR deletions may affect the level of virus replication and attenuation. Again, additional studies to explore the role of the HVR in HEV attenuation, which is not within the scope of this study, will provide more insights into the role of the HVR in the biology and pathogenesis of HEV.

The patterns of viremia and fecal virus shedding in experimentally infected pigs and chickens (Tables 2.2 and 2.3) are somewhat different from that observed in HEV-infected humans. In humans, viremia usually precedes fecal virus shedding, whereas in the pig and chicken studies fecal virus shedding was detected prior to viremia, which is consistent with our previous animal studies (5, 16). It is believed that tissues in the gastrointestinal tract are the initial sites of swine HEV and avian HEV replication, and thus, the virus is excreted to the feces before entering the bloodstream. In fact, it has recently been demonstrated that after oral injection or intravenous injection, swine HEV and avian HEV first replicate in various gastrointestinal tissues in chickens and pigs before reaching the target organ, the liver, via the bloodstream (6, 59), and this may explain why fecal virus shedding precedes viremia in HEV-infected pigs and chickens.

Since ORF1 contains domains essential for HEV replication, proper folding of the encoded polyprotein is essential for its role in virus replication either individually or by interacting with host alleles. The results from our *in vitro*, as well as *in vivo*, animal studies showed that the deletions in the HVR did not eliminate the viability of the virus, and thus, the polyprotein encoded by ORF1 appears to be properly folded in viable mutants. The HVR, aa 747

to 761 of genotype 1 human HEV, aa 557 to 585 and 612 to 641 of avian HEV, and aa 712 to 765 of genotype 3 swine HEV, apparently has no major effect on the host-mediated processing of the polyprotein, as the mutant viruses are viable and infectious in animals. Since unneeded sequences in virus genomes normally are lost rapidly during *in vivo* replication, it is possible that the HVR, although not essential for virus infectivity, may play a biological role in HEV pathogenesis. In fact, the results from the animal studies with limited numbers of pigs and chickens suggested that deletions of a larger or nearly complete HVR from the HEV genome apparently attenuated the virus. Therefore, additional studies with larger numbers of animals are warranted to fully evaluate the biological role of HVR in HEV replication and pathogenesis.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (AI050611 and AI065546). We thank Suzanne Emerson and Robert Purcell at the Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, MD, for generously providing us the genotype 1 human HEV (Sar55 strain) infectious clone and a subclone of the Huh7 liver cell line. We also thank Padma Billam for her technical assistance.

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Figure 2.1

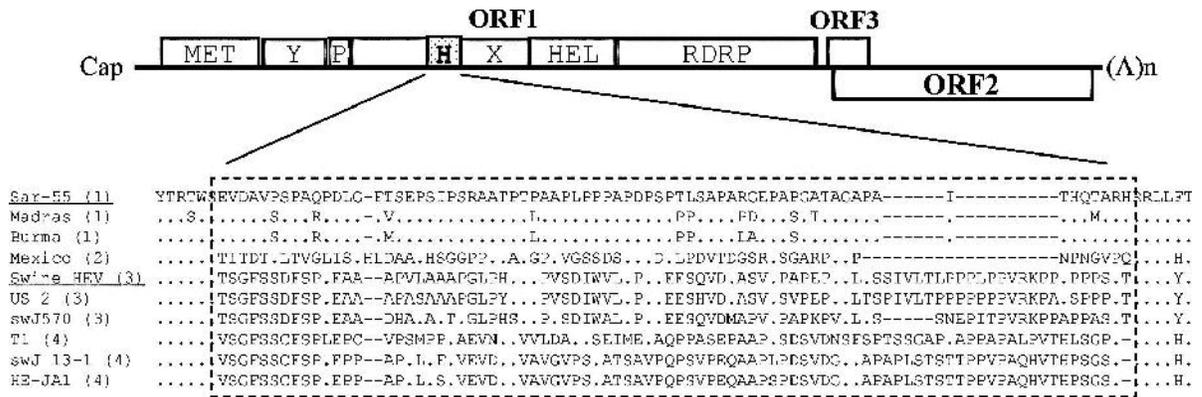


Figure 2.1. Schematic diagram showing the relative positions of the HVR of ORF1 from representative isolates in four major genotypes of mammalian HEV (genotypes 1 to 4, in parentheses), along with putative functional domains: MET, methyltransferase; P, papain-like cysteine protease; Y, Y domain; H, HVR; X, X domain; HEL, helicase; RDRP, RNA-dependent RNA polymerase

Figure 2.2

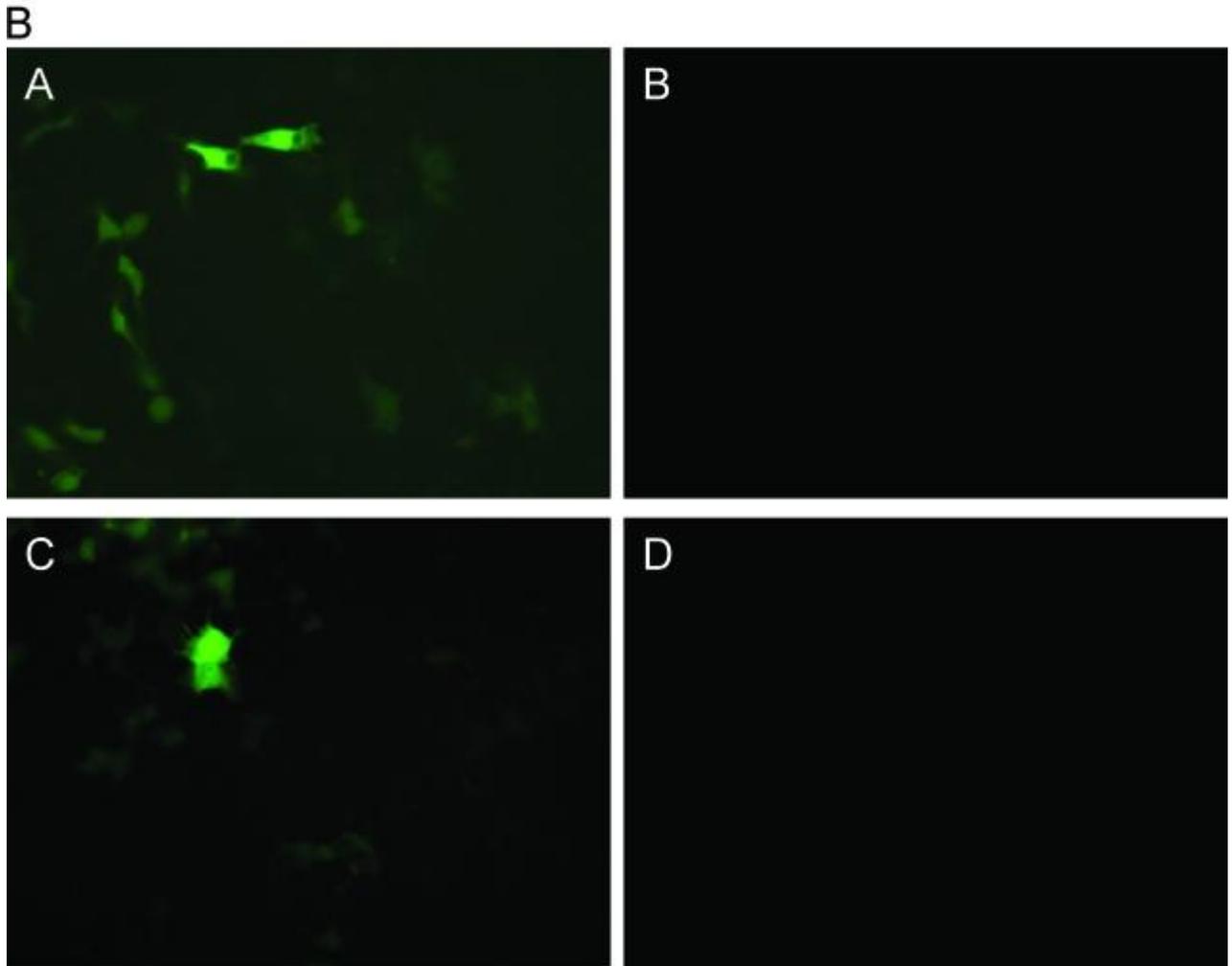
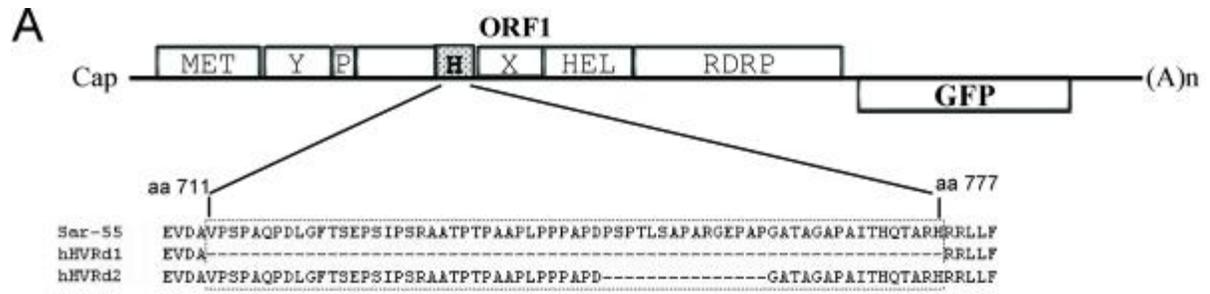


Figure 2.2. Schematic diagram showing the relative positions of in-frame HVR deletions in the HVR of genotype 1 human HEV and fluorescence microscopy of Huh7 cells. (A) Schematic diagram showing the HVR (aa 707 to 777) in ORF1 of the genotype 1 human HEV (strain Sar55) replicon expressing EGFP. MET, methyltransferase; P, papain-like cysteine protease; Y, Y domain; H, HVR; X, X domain; HEL, helicase; RDRP, RNA-dependent RNA polymerase. The amino acid sequence of each HVR deletion mutant is aligned with that of the wild-type Sar55 HEV replicon to show the relative positions of the in-frame amino acid deletions: mutants hHVRd1 (aa 711 to 777) and hHVRd2 (aa 747 to 761). (B) Fluorescence microscopy of Huh7 liver cells at 6 days posttransfection with similar amounts of capped RNA transcripts from the wild-type Sar55 replicon with the EGFP gene (A), HVR deletion mutants hHVRd1 (B) and hHVRd2 (C), and mock-transfected cells (D).

Figure 2.3

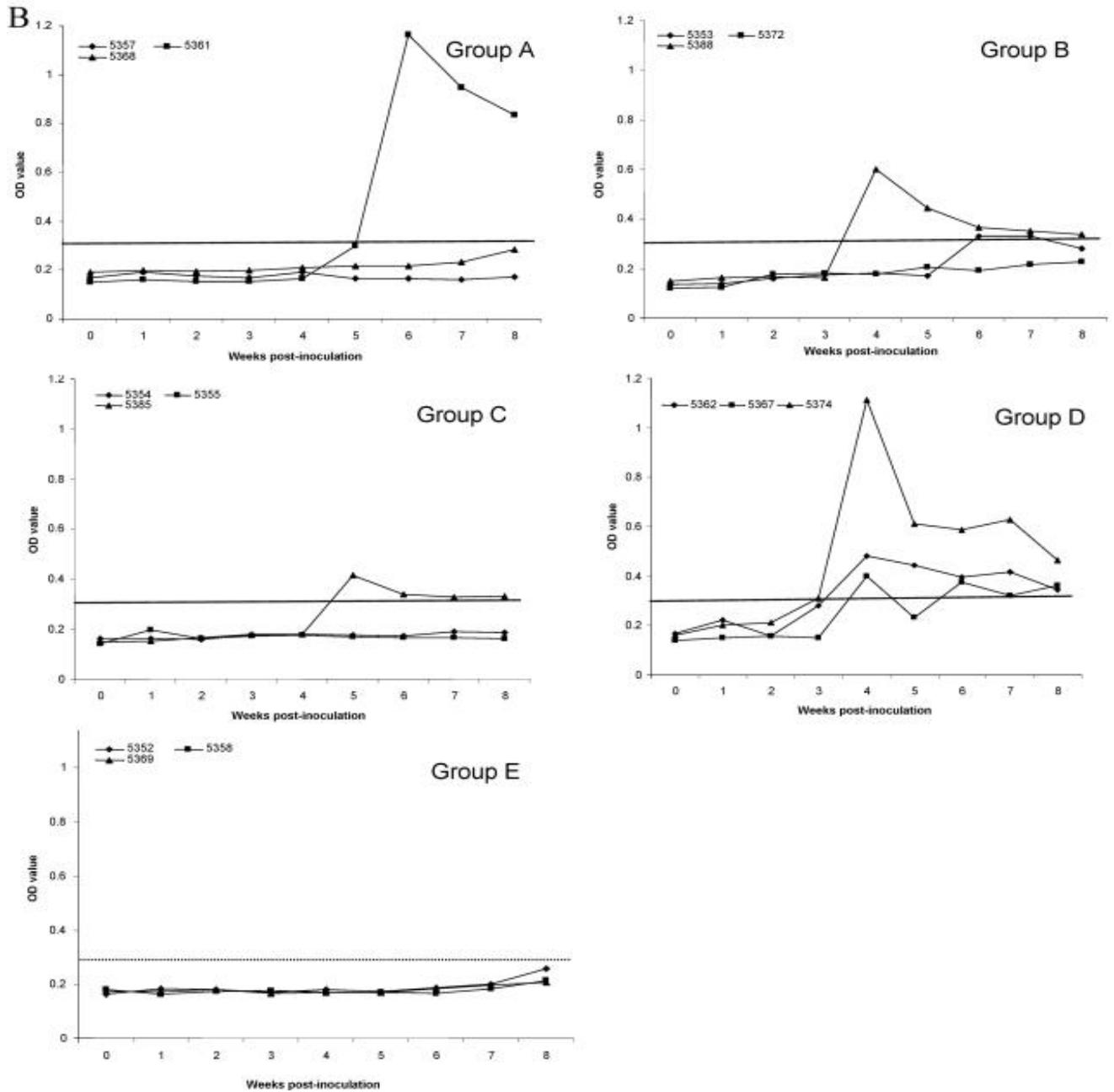
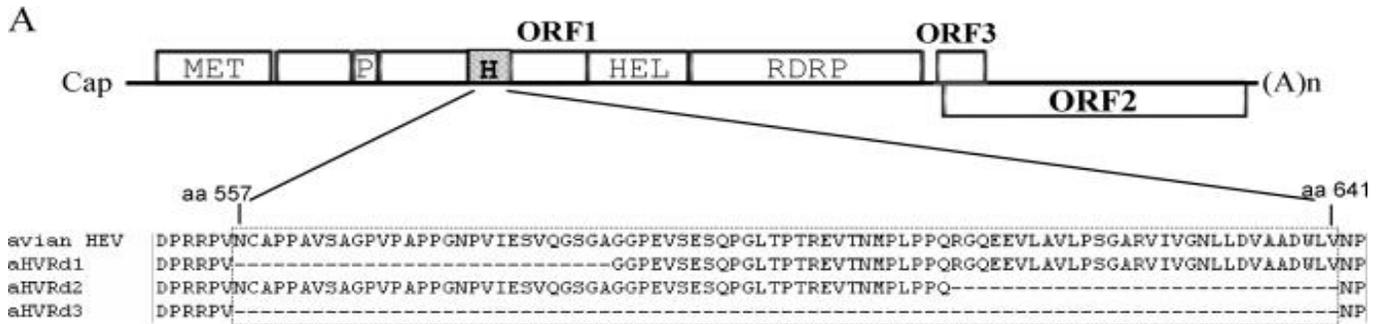


Figure 2.3. Schematic diagram showing the relative positions of in-frame HVR deletions in the HVR of avian HEV strain, and seroconversion to IgG anti-HEV in chickens. (A) Schematic diagram showing the HVR (aa 557 to 641) in ORF1 of avian HEV, along with putative functional domains: MET, methyltransferase; P, papain-like cysteine protease; H, HVR; HEL, helicase; and RDRP, RNA-dependent RNA polymerase. The amino acid sequence of each HVR deletion mutant is aligned with that of the wild-type strain of avian HEV to show the relative positions of the in-frame amino acid deletions: mutants aHVRd1 (aa 557 to 585), aHVRd2 (aa 612 to 641), and aHVRd3 (aa 557 to 641). (B) Seroconversion to IgG anti-HEV in chickens inoculated with capped RNA transcripts from the wild-type avian HEV infectious clone and its derived HVR deletion mutants. IgG anti-HEV was plotted as the ELISA optical density (OD) (A_{405}), and the ELISA cutoff value was 0.30. Chickens 5357, 5361, and 5368 were each inoculated with RNA transcripts from HVR deletion mutant aHVRd1 (group A); chickens 5353, 5372, and 5388 with RNA transcripts from mutant aHVRd2 (group B); and chickens 5354, 5355, and 5385 with RNA transcripts from mutant sHVRd3 (group C). Chickens 5362, 5367, and 5374 (group D) were each inoculated with RNA transcripts from the wild-type avian HEV infectious cDNA clone as positive controls, and chickens 5352, 5358, and 5369 (group E) were intrahepatically inoculated with PBS buffer as negative controls.

Figure 2.4

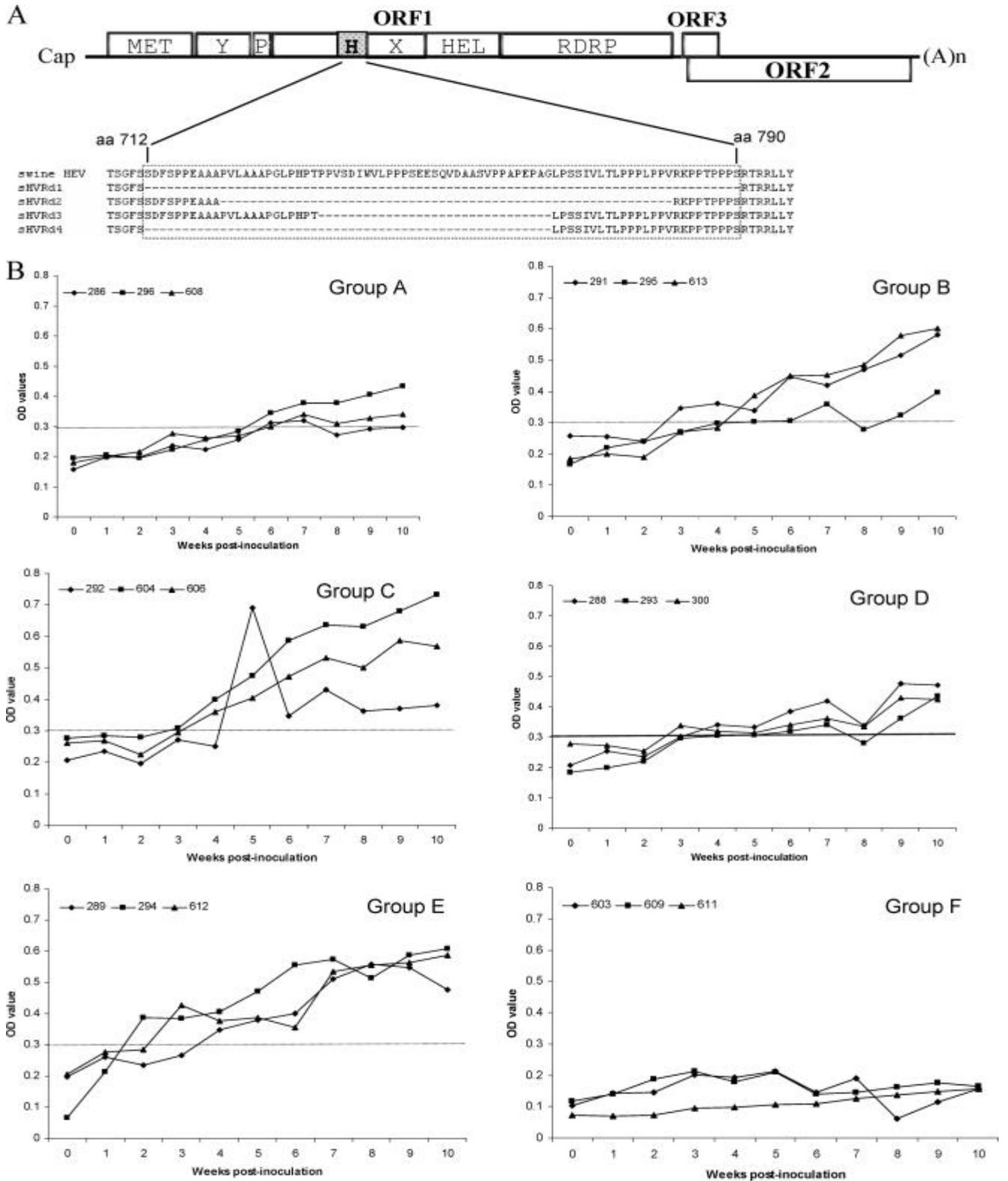


Figure 2.4. Schematic diagram showing the relative positions of in-frame HVR deletions in the HVR of genotype 3 swine HEV strain, and seroconversion to IgG anti-HEV in pigs. (A) Schematic diagram showing the HVR (aa 707 to 790) in ORF1 of the genotype 3 swine HEV. MET, methyltransferase; P, papain-like cysteine protease; Y, Y domain; H, HVR; X, X domain; HEL, helicase; and RDRP, RNA-dependent RNA polymerase. The amino acid sequence of each HVR deletion mutant is aligned with that of the wild-type strain of the genotype 3 swine HEV to show the relative positions of the in-frame amino acid deletions: mutants sHVRd1 (aa 712 to 790), sHVRd2 (aa 722 to 781), sHVRd3 (aa 735 to 765), and sHVRd4 (aa 712 to 765). (B) Seroconversion to IgG anti-HEV in pigs inoculated with capped RNA transcripts from the wild-type swine HEV infectious clone and its derived HVR deletion mutants. IgG anti-HEV was plotted as the ELISA optical density (OD) (A_{405}), and the ELISA cutoff value was 0.30. Pigs 286, 296, and 608 were each inoculated with capped RNA transcripts from HVR deletion mutant sHVRd1 (group A); pigs 291, 295, and 613 with RNA transcripts from mutant sHVRd2 (group B); pigs 292, 604, and 606 with RNA transcripts from mutant sHVRd3 (group C); pigs 288, 293, and 300 with RNA transcripts from mutant sHVRd4 (group D); and pigs 289, 294, and 612 with RNA transcripts from wild-type genotype 3 swine HEV infectious clone pSHEV-3 (group E). Pigs 603, 609, and 611 were intrahepatically inoculated with PBS buffer as negative controls

Table 2.1. Oligonucleotide primers used for construction of HVR deletion mutants, as well as for PCR and sequencing.

Primer	Sequence (5' →3')
For construction of avian HEV HVR deletion mutants	
AvF	CCCGTAACTGCGCACCACCGGCCG
Avf	CCCGTTAACGGCGGACCTGAGGTCA
AvR	CGGGTTAACAAGCCAGTCGGCGGCA
Avr	CGGGTTAACCTGTGGCGGCAAGGGC
For construction of swine HEV HVR-deletion mutants	
SwF	GACATCGCCGCTCGAGCCTCCCGCCTAA
Swf1	ACATCTGGCTTTTCTCGACTCGTCGTCTC
Swf2	CCTGAGGCGGCCGCCCGTAAGCCACCAACA
Swf3	CTGCCCCACCCTACCTTACCCAGCTCCATT
Swf4	ACATCTGGCTTTTCTTTACCCAGCTCCATT
SwR	GGGACCTGGTATATACCCGAGCCTAGAA
Swr1	AGAAAAGCCAGATGTTGACCAAGTCCGGGT
Swr2	GGCGGCCGCCTCAGGGGGGGAGAAATCGCT
Swr3	GGTAGGGTGGGGCAGCCCCGGGGCAGCGGC
For construction of genotype 1 human HEV replicon HVR-deletion mutants	
HuF	GGGAGCATGCTCAGAAGTTTATAACACGCC
Huf1	TCGGAGGTTGATGCTCGCCGCCTGCTCTTT
Huf2	CCCCCTGCACCGGATGGCGCTACCGCCGGG
HuR	GTACCTCTGGTAAAATGCATGACAGAGCCC
Hur1	AGCATCAACCTCCGACCAAGTGCGGGTGTA
Hur2	ATCCGGTGCAGGGGGGGGTAGAGGGGCCGC
For sequencing of the HVR region from viruses recovered from pigs and chickens	
Av N1	TTACCATTGACTTTGAACGGCG
Av N2	CCGGGCTGATGGTCTCGATTAG
Av N3	GCTTGTGCATTGACGATTTCCC
Av N4	CAATAGGTTACCCACGATGACG
Sw N1	CAGGTCAGGATTTTCATCTAATGG
Sw N2	GCCTCACGCATAATGAACTCAG

Table 2.2. Detection of avian HEV RNA from fecal and serum samples of SPF chickens intrahepatically injected with RNA transcripts from the avian HEV cDNA clones^a

Group	Chicken no.	Result (fecal/serum) at week p.i.									
		0	1	2	3	4	5	6	7	8	
A (aHVRd1)	5357	-/-	-/-	-/-	+/-	-/-	-/-	+/-	-/-	-/-	
	5361	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	-/-	
	5368	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
B (aHVRd2)	5353	-/-	-/-	-/-	+/+	-/-	-/-	-/-	-/-	+/-	
	5372	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	5388	-/-	-/-	+/-	+/+	-/+	-/+	-/+	-/-	-/-	
C (aHVRd3)	5354	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	5355	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	5385	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
D (positive control group)	5362	-/-	-/-	+/-	+/+	-/-	-/-	-/-	-/-	-/-	
	5367	-/-	-/-	-/-	-/-	+/-	-/-	-/-	-/-	-/-	
	5374	-/-	-/-	-/+	+/+	-/-	-/-	-/-	-/-	-/-	
E (Negative control group)	5352	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	5358	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	5369	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	

^a Positive (+) or negative (-) samples at the indicated week p.i. in SPF chickens intrahepatically inoculated with capped RNA transcripts of avian HEV HVR deletion mutants, as well as the wild-type avian HEV infectious clone.

Table 2.3. Detection of swine HEV RNA from fecal and serum samples of SPF pigs intrahepatically injected with RNA transcripts from the swine HEV cDNA clones^a

Group	Pig no.	Result (fecal/serum) at week p.i.											
		0	1	2	3	4	5	6	7	8	9	10	
A (sHVRd1)	286	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	296	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	608	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
B (sHVRd2)	291	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-
	295	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-
	613	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
C (sHVRd3)	292	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	604	-/-	-/-	-/-	+/+	-/-	+/-	+/+	+/-	+/-	-/-	+/-	
	606	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
D (sHVRd4)	288	-/-	-/-	+/-	+/-	+/-	+/+	+/-	+/-	+/-	+/-	+/-	-/-
	293	-/-	-/-	+/-	+/-	+/-	-/-	-/-	-/-	+/-	-/-	+/-	
	300	-/-	-/-	-/-	+/-	+/-	+/-	+/-	-/-	+/-	-/-	+/-	
E (positive control group)	289	-/-	-/-	-/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	
	294	-/-	-/-	+/-	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-/-	
	612	-/-	-/-	-/-	-/-	+/-	+/-	-/-	+/-	-/-	+/-	-/-	
F (Negative control group)	603	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	609	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	
	611	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	

^a In samples that were positive (+) or negative (-) at the indicated week p.i. in SPF pigs intrahepatically inoculated with capped RNA transcripts of swine HEV HVR deletion mutants, as well as the wild-type infectious clone.

Chapter 3

Deletion analysis of the hypervariable region (HVR) in the open reading frame 1 (ORF1) of the hepatitis E virus: Effects on viral replication

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and X. J. Meng

ABSTRACT

Hepatitis E virus (HEV) is a major cause of acute hepatitis in many developing countries and is also endemic in some industrialized countries. The positive-strand RNA genome of HEV consists of a hypervariable region (HVR) in the ORF1. We recently demonstrated that HEV tolerated deletions in the HVR for its infectivity, suggesting a functional flexibility in the amino acid sequence of HVR. Although the HVR is a flexible domain with residues that are not absolutely required for HEV infectivity, it may interact with host factors to play more subtle roles in either viral replication or pathogenesis. To further investigate the role of HVR in viral replication and to quantify viral RNA synthesis *in vitro*, we first constructed a HEV replicon expressing a luciferase reporter using the genotype 1 human HEV. Subsequently, a series of deletion mutants with various lengths of deletion in the HVR were constructed. Upon transfection of the deletion mutants into Huh7 human liver cells, the intracellular luciferase activity which is a measure of viral RNA replication was compared between wildtype HEV replicon and HVR-deletion mutants at 4 days post-transfection. Though the HVR-deletion mutants are replication-competent in Huh7 cells, the replication levels were significantly reduced when compared to that of wildtype replicon, thus indicating the role of HVR in efficient HEV replication. To further confirm the results obtained from the genotype 1 human HEV, we subsequently constructed a distantly-related non-mammalian avian HEV replicon expressing luciferase reporter and analyzed the relative replication levels of avian HEV replicon expressing luciferase and three derived HVR-deletion mutants in LMH chicken liver cells. The results were

similar to those obtained with the genotype 1 human HEV replicons, as significant reduced levels of avian HEV replications were observed for deletion mutants compared to that of wild-type avian HEV replicon, including the mutant with complete HVR deletion. To determine the effect of complete HVR deletion on the avian HEV replication levels *in vivo*, we conducted an infection study in specific-pathogen-free (SPF) chickens. Complete HVR deletion mutant was constructed using the backbone of full-length avian HEV infectious clone and was shown to be viable when transfected into LMH cells. The capped RNA transcripts from complete HVR deletion-mutant as well as the wildtype HEV were intrahepatically inoculated into the livers of chickens to evaluate the replication ability of the complete HVR deletion mutant. Evidence of HEV replication was not detected in any of the chickens inoculated with complete HVR-deletion mutant whereas the chickens inoculated with the wildtype HEV were infected, leading to the speculation that drastic reduction in replication efficiency of the complete HVR deletion as observed *in vitro* have contributed for the inability of complete HVR deletion mutant to establish a productive infection. Our results suggest that HVR contains critical amino residues that may interact with host factors and influence HEV replication efficiency, and the amino acid residues which are absolutely required to establish a productive infection or for the HEV survival needs to be determined in the future.

INTRODUCTION

Hepatitis E virus (HEV), the causative agent of enterically transmitted acute viral hepatitis, is classified in the genus *Hepevirus* of the family *Hepeviridae* (37). Hepatitis E is an important public health disease in both developing and industrialized countries (4, 7, 18, 35). In recent years, the bulk of the evidence documented by scientists suggests that hepatitis E is a zoonotic disease and animal reservoirs exist for HEV (40, 51, 88, 89, 92, 95, 133, 153).

The 7.2 kb genome of HEV contains three open reading frames: ORF1, ORF2, and ORF3, numbered according to their gene order (129). ORF1 encodes nonstructural polyproteins, which has functional domains essential for replication of HEV (72, 129). The two subgenomic structural proteins, including a major capsid protein of approximately 660 amino acids, and a multifunctional protein of 56 kDa (aa 112-607) are encoded by ORF2 and ORF3, respectively (26, 96, 97, 113, 141, 150). The methyltransferase and guanylyltransferase activities function in capping of the viral plus-strand RNA (84), the role of RNA-dependent RNA polymerase (RdRp)

in RNA synthesis (3), and the helicase-associated 5' -triphosphatase (67), and NTPase and RNA duplex-unwinding activities have been demonstrated(66). The RNA-dependent RNA polymerases of HEV (32, 46), including mammalian and avian HEV strains, contains the conserved GDD sequence motif, the mutation of which affects host cell-dependent replication.

The class of positive-strand RNA viruses includes pathogens that cause a significant number of human, animal and plant diseases. Different groups of viruses in this class encode related nonstructural proteins that are known to be involved in viral RNA replication. Hepatitis E virus (HEV), for example, encodes replication factors that have similarities with the rubella virus (RubV), and alpha viruses (72). The organization of the putative conserved functional domains in viral polyproteins of HEV, closely related to rubella virus (RubV) and phylogenetically distant alphaviruses, are collinear and differ in the mutual arrangement of individual domains in the cluster protease-X-helicase. The highly conserved "X" domain that flanks the papain-like protease domains of each virus, is preceded by a prolin-rich hinge region that may constitute a flexible hinge between the X domain and the upstream domains (72). The hypervariable region (HVR) of HEV overlaps with its prolin-rich region (108). We recently demonstrated that HEV can tolerate deletions in HVR for its infectivity both *in vitro* and *in vivo* and amino acid residues in this region are dispensable for replication (108). Previously, based on the sequence comparison of only two strains of avian HEV and mammalian HEVs, we predicted that amino acids 557 to 641 are hypervariable (108). However, since that initial publication, two new genotypes of avian HEV have been identified from chickens worldwide (17, 158), and the availability of the additional avian HEV sequences from the two new genotypes now allows us to more precisely predict the hypervariable region of avian HEV which spans amino acids 557 to 603.

To further investigate the role of HVR on HEV replication, we first constructed a series of mutants with overlapping deletions in the N-terminal region, central region, and C-terminal region of HVR, using the backbone of the genotype 1 human HEV luciferase replicon and analyzed the effects of these deletions on viral RNA replication in Huh7 cells. We found that replication levels of the HEV mutants were markedly reduced in Huh7 cells, suggesting a role for HVR in efficient replication of HEV. To further verify the results, we constructed HVR deletion mutants using a non-mammalian avian HEV. Similar effects of HVR deletions on viral replication were observed when deletion-mutants of avian HEV luciferase replicon were tested in

LMH cells. The infectivity of complete HVR deletion-mutant of avian HEV was also tested in specific-pathogen-free (SPF) chickens. Although, complete HVR deletion-mutant of avian HEV is replication-competent in LMH cells, complete deletion of HVR (aa Δ 557-603) resulted in loss of infectivity in SPF-chickens.

MATERIALS AND METHODS

Generation of genotype 1 human HEV plasmid constructs: The HEV replicon expressing fire fly luciferase was constructed by replacing the amino terminus of the ORF2 capsid gene (nt 5148 to 5816) of the full-length infectious cDNA clone of a genotype 1 human HEV (strain Sar55), pSK-HEV-2 (reference, kindly provided by Drs Suzanne U. Emerson and Robert H. Purcell of NIH, Bethesda, MD). The fire fly luciferase gene was inserted in frame with the ORF2 initiation codon. The 5' NCR, ORF1, and 3' NCR in the luciferase replicon are intact, with part of the carboxy terminus of ORF2 fused to the luciferase gene. Briefly, the nucleotides from 5014 to 5147 was amplified from the infectious cDNA clone by using the primer set HuLP1/HuLP3, and the fire fly luciferase gene was amplified from pGL4.10[luc2] Vector (Promega) from nt 100 to 1752 by using the primer set HuLP4/HuLP2. The PCR products amplified were then used in the fusion PCR with primer set HuLP1/HuLP2 (Table 3.1). The fusion product was substituted for the *SphI*-to-*EcoRI* region in the infectious cDNA clone. A null replication mutant of genotype 1 human HEV replicon, pSK-GAA-luc, was constructed by mutating conserved GDD motif of RdRp to GAA using quick-change mutagenesis (Stratagene) using the primer HuGAAF, and its complement HuGAAR (corresponding to nucleotides 4661–4692 of ORF1).

Nine HVR deletion mutants of the genotype 1 human HEV replicons were constructed using fusion PCRs (Figure 3.1). Amino acid residues 711 to 725, 711 to 740 and 711 to 750, corresponding to nucleotides (nt) 2131 to 2175, 2231 to 2220 and 2131 to 2250 were deleted in the N-terminal region of HVR to generate the HVR deletion mutants H51-luc, H52-luc and H53-luc, respectively. The two fragments used for fusion PCR were first amplified with the primer sets Hu F/Hu r1 and Hu f1/Hu R (Table 3.1) for the mutant H51-luc, primers Hu F/Hu r2 and Hu f2/Hu R for the mutant H52-luc, and primers Hu F/Hu r3 and Hu f3/Hu R for the mutant H53-luc (Table 3.1). Amino acid residues 729 to 759 and 721 to 766 and 716 to 771 corresponding to nucleotides (nt) 2185 to 2277, 2161 to 2298 and 2146 to 2313, were deleted in the central HVR region to construct the HVR deletion mutants Hm1-luc, Hm2-luc and Hm3-luc, respectively. The

two fragments used for fusion PCR were first amplified with the primer sets Hu F/Hu r4 and Hu f4/Hu R for the mutant Hm1-luc, primers Hu F/Hu r5 and Hu f5/Hu R for the mutant Hm2-luc, and primers Hu F/Hu r6 and Hu f6/Hu R for the mutant Hm3-luc. Amino acid residues 761 to 775, 746 to 775 and 736 to 775, corresponding to nucleotides (nt) 2287 to 2325, 2242 to 2325 and 2212 to 2325, were deleted in the C-terminal region of HVR to produce the HVR deletion mutants H31-luc, H32-luc and H33-luc, respectively. The two fragments used for fusion PCR were first amplified with the primer sets Hu F/Hu r7 and Hu f7/Hu R for the mutant H31-luc, primers Hu F/Hu r8 and Hu f8/Hu R for the mutant H32-luc, and primers Hu F/Hu r9 and Hu f9/Hu R for the mutant H33-luc. The PCR products amplified from each mutant were then used in the fusion PCR with primer set Hu F/Hu R (Table 3.1). To produce the two HVR deletion mutants, the fusion product was purified with QIAEX II gel extraction kit (Qiagen), digested with *SphI* and *NsiI*, and ligated into the backbone of the genotype 1 human HEV Luciferase replicon from which the *SphI-NsiI* region had already been deleted.

Generation of avian HEV plasmid constructs: To further verify the role of HVR in HEV replication from the results obtained with the genotype 1 human HEV replicons, we selected the distantly-related non-mammalian avian HEV and constructed avian HEV replicon expressing fire fly luciferase by replacing the structural component of virus with the luciferase reporter gene. fire fly luciferase gene was inserted in frame with the start codon of ORF2, replacing the nucleotides 3752 to 6090 of the full-length infectious cDNA clone of avian HEV strain, pT7-aHEV (reference, Gene bank accession number AY535004). Avian HEV genome fragment (nt 3752 to 4709) and fire fly luciferase gene (nt 100 to 1752) were amplified using the primer sets AvEcoRVF/AvBsmBIr and AvBsmBIr/AvSalIR respectively. The fragments were ligated after digesting with the *BsmBI* restriction enzyme. The ligated product was finally amplified with the primers AvEcoRVF and AvSalIR. The fusion product was then substituted for the *EcoRV*-to-*Sall* region in the infectious cDNA clone. A null replication mutant of avian HEV replicon, AvGAA-luc, was constructed by mutating conserved GDD motif of RdRp to GAA using quick-change mutagenesis (Stratagene) using the primer AvGAAF, and its complement AvGAAR (corresponding to nucleotides 4182–4219).

In-frame amino acid deletions $\Delta 557-603$, $\Delta 566-595$, and $\Delta 573-587$ were engineered into the backbone of the avian HEV replicon corresponding to the nucleotide positions (nt) 1693 to 1833, 1720 to 1809, and 1741 to 1785 to generate HVR deletion mutants Avm1-luc, Avm2-luc,

and Avm3-luc, respectively. A PCR fragment was first amplified using the primers Av-p1A and AvR to engineer a HVR deletion that requires further mutagenesis to construct complete HVR deletion-mutant, Avm1-luc. The two fragments used for fusion PCR were first amplified with the primer sets AvFF / Av-p2A and Av-p2B /AvR for the mutant Avm2-luc, and AvFF/ Av-p3A and Av-p2B/AvR for the mutant Avm3-luc (Table 3.2). PCR products amplified from each mutant were then used in the fusion PCR with primer set AvFF/AvR (Table 3.2). The PCR product initially amplified with the primer set Av-p1A/AvR, and the fusion PCR products were purified, digested with Hpa I, and ligated into the backbone of the avian HEV luciferase replicon. A final site-directed mutagenesis was performed on Avm1-luc, using the primer Av-p1F and its complement Av-p1R, to engineer the complete HVR deletion. Amino acid deletion $\Delta 557-603$ was also engineered into the full-length infectious cDNA clone of avian HEV to construct the complete HVR deletion mutant, cAvm1, for the *in vivo* chicken study.

***In vitro* RNA synthesis.** The plasmid constructs with the backbone of genotype 1 human HEV luciferase replicon were linearized with a *Bgl II* restriction site directly downstream of the poly(A) sequence, and the constructs of avian HEV luciferase replicon and full-length infectious cDNA clone were linearized using a *Xho I*, followed by phenol-chloroform extraction and ethanol precipitation. *In vitro* RNA transcripts were subsequently produced using the mMessage mMachine T7 Kit (Ambion) according to the manufacturer's instructions. For synthesis of 5'-capped RNA transcripts, 1 μ g of linearized plasmid was transcribed at 37°C in a 20- μ l reaction mixture containing 2 μ l 10X reaction buffers, 10 μ l 2X nucleoside triphosphate/Cap, 2 μ l enzyme mixture, and an additional 1 μ l 30 mM GTP stock for capping. The integrity of transcripts was analyzed by electrophoresis in 1% agarose gels. The yields of the *in vitro*-generated RNA transcripts were quantified using the Quant-iT RiboGreen RNA assay Kit (Molecular Probes). To remove template DNA, 0.25 μ l of TURBO DNase I (Ambion) was added to 5 μ l of the transcription reaction mixture and incubated at 37°C for 15 minutes. The concentration of each DNase I digested sample was measured as per manufacturer's instructions.

***In vitro* transfection of cells.** The *in vitro*-generated RNA transcripts were used for transfection of a subclone of Huh7 human liver cells S10-3 cells (a gift of Dr. Suzanne U. Emerson, NIH, Bethesda, MD) and LMH chicken liver cells. RNA transcripts synthesized from the T7 promoter of a Renilla luciferase vector (pRL-TK, Promega) was used for normalization and as an internal control.

For the transfection of Huh7 cells with RNA transcripts generated from the genotype 1 human HEV replicon, 1 µg of transcribed RNAs from each deletion-mutant construct and 50 ng of RNA from pRL-TK vector were mixed with 5 µl of DMRIE-C (Invitrogen) and 500 µl of Opti-MEM (Invitrogen) to transfect Huh7 cells seeded at 60% confluency in one well of a 12-well plate. Transfections were performed in quadruplicates for each sample. After 5 hours of incubation at 34.5°C, the transfection mixture was replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and the plates were again incubated at 34.5°C.

LMH cells, which support the replication of avian HEV (56), were transfected at approximately 85% confluency with RNA transcripts generated from avian HEV replicon constructs in a 12-well plate using Lipofectamine LTX kit (Invitrogen). RNA transcripts at a concentration of 2µg were mixed with 1.5 µl of Plus reagent (Invitrogen) in 100 µl of Opti-MEM and incubated at room temperature for 5 minutes. Three microliters of Lipofectamine LTX reagent was added to the mix and incubated for additional 15 minutes at room temperature. The mixture containing the RNA transcripts, Plus Reagent and Lipofectamine mixtures were then added to pre-washed LMH cells covered with 400 µl of Opti-MEM. After incubation at 37 °C for 4 hours, the transfection mixture was replaced with Waymouth's MB 752/1 medium supplemented with 10% fetal bovine serum and 5% chicken serum, and the incubation was continued at 37 °C.

Luciferase assay. Huh7 cells at 4 days post-transfection and LMH cells at 5 days post-transfection were washed with phosphate-buffered saline (PBS) and cell lysates were prepared from transfected cells. The cell lysates were centrifuged briefly and 20 µl of the supernatants were used for the luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a *Tecan* GENios Microplate Reader.

Immunofluorescence assay (IFA). Five days post-transfection, the LMH cells were trypsinized and re-plated on 24-well plate. On day 6, the LMH cells were rinsed with PBS, fixed with a solution containing 70 % acetone and 30 % ethanol on ice for 1 hour, and air dried. A 1:500-diluted anti-avian HEV convalescent serum from a SPF chicken experimentally infected with avian HEV was added to the fixed cells and incubated for 1 hour at 37 °C. After washing with cold PBS (1X) with 0.1% Tween-20, 1:1500-diluted fluorescein-labelled goat anti-chicken IgG

(KPL) was added and incubated at 37 °C for 30 min. The wells were then washed with PBS, and viewed under a fluorescence microscope.

Intrahepatic inoculation of chickens with capped RNAs transcript from avian HEV clones

To test the infectivity and *in vivo* replication competency of the avian HEV complete HVR-deletion mutant, a surgical procedure was used to visually expose the livers for intrahepatic inoculation of chickens with capped RNA transcripts from the wild-type avian HEV infectious clone and the complete HVR-deletion avian HEV mutant (56). Briefly, 30, 4-week-old, SPF chickens that were negative for avian HEV RNA and antibodies were divided into three groups (groups A through C), with 10 chickens in each group (Figure 3.4). The RNA transcripts were injected into two different sites in each liver, with approximately 200 µl per injection site. Ten chickens in group A (198, 185, 152, 146, 164, 162, 101, 120, 170, and 148) were each injected with 400 µl of RNA transcripts from mutant cAvm1. Chickens 20, 467, 40, 34, 56, 60, 64, 66, 69, and 80 in group B were each injected with RNA transcripts from the wild-type avian HEV infectious clone and served as positive controls. The ten chickens in group C (443, 404, 477, 469, 496, 402, 430, 494, 417, and 498) were injected similarly with PBS buffer as negative controls (Figure 3.4). Fecal swabs and sera were collected from each chicken prior to inoculation and weekly thereafter and tested by RT-PCR for avian HEV RNA. Weekly serum samples were also tested by enzyme-linked immunosorbent assay (ELISA) for seroconversion to avian HEV antibodies as previously described (55, 57). All inoculated chickens were necropsied at 5 weeks postinoculation (p.i.).

RESULTS

To examine the possible contribution of hypervariable region (HVR) to HEV replication, a series of deletion mutations were introduced into the infectious cDNA clones of genotype 1 human HEV strain, pSK-HEV2 (38) and avian HEV strain, pT7-aHEV (56) using standard recombinant DNA technologies. Deletions were generated by PCR-based technologies, and amplified fragments were cloned into parental replicon vector or full-length infectious cDNA clone by using appropriate restriction sites. The ability of altered HVR to support viral RNA

replication was tested by transfecting Huh7 cells and LMH cells with capped RNA transcripts from deletion mutants of human HEV and avian HEV, respectively. The effect of complete HVR deletion on the replication and infectivity of avian HEV in chickens was also tested.

Effects of N-terminal deletions of the HVR on HEV RNA replication. The replication levels of HVR N-terminal deletion mutants H51-luc ($\Delta 711-725$), H52-luc ($\Delta 711-740$), and H53-luc ($\Delta 711-750$) were compared with that of the wild-type HEV in Huh7 cells. Deletions at the N-terminal region of HVR did not abolish viral replication but substantially reduced the replication levels of viral RNA, as evidenced by the significantly reduced luciferase activity in the deletion mutants (Figure 3.2). Furthermore, mutants with larger deletions replicated at lower levels than those of mutants with smaller deletions. The percentage of reduction in replication levels when compared to wild-type HEV for the mutants H51-luc ($\Delta 15aa$), H52-luc ($\Delta 40aa$), and H53-luc ($\Delta 50aa$) are approximately 85%, 50%, and 13%, respectively.

Effects of deletions in central region of HVR on HEV RNA replication. Mutants Hm1-luc, Hm2-luc, and Hm3-luc were tested in Huh7 cells to determine if the deletions of amino acid residues in the central region of HVR have distinct effects on viral replication. Analogous to the results of N-terminal deletions of HVR, various length deletions in the central region of HVR also showed a gradient of reduced viral replication activity, where mutants with larger deletions replicated at lower levels than those of mutants with smaller deletions (Figure 3.2). The replication level of each mutant was drastically reduced as evidenced by the significantly reduced luciferase activity. When compared to the wildtype HEV, the replication levels for the mutants Hm1-luc, Hm2-luc, and Hm3-luc were only at 34.5%, 13%, and 2.5%, respectively (Figure 3. 2), indicating deleterious effects of deletions in the central region of the HVR on HEV RNA replication.

Effects of C-terminal deletions of HVR on HEV RNA replication. When the replication levels of the C-terminal HVR deletion mutants H31-luc, H32-luc, and H33-luc were compared to that of the wild-type HEV, the replication levels were reduced only about two-fold and the length of the deletions in the C-terminal region of the HVR appeared to have no significant gradient effect. Differences in replication levels among deletion mutants H31-luc, H32-luc, and H33-luc were small, with luciferase activities of 62%, 44%, and 53% for respective mutants, suggesting the relative flexibility of the N-terminal region of HVR (Figure 3.2).

Effects of various lengths of HVR deletions on avian HEV RNA replication. To further verify the role of HVR in HEV replication observed in genotype 1 human HEV, we subsequently constructed a HEV replicon from a distant non-mammalian family member, avian HEV, and evaluated the replication efficiency of three deletion mutants of avian HEV in LMH chicken liver cells. Similar to the results we observed for the deletions in N-terminal and central region of HVR for the genotype 1 human HEV, gradient reduction in the viral replication activity was observed for the mutants Avm1-luc, Avm2-luc, and Avm3-luc in LMH cells (Figure 3.3). Mutant Avm1-luc with a larger deletion in the HVR region replicated at lower level (28%) compared to those of mutants Avm2-luc (36%) and Avm3-luc (43%) with smaller deletions.

Complete HVR deletion mutant of avian HEV is non-infectious *in vivo*. In an attempt to determine whether complete HVR deletion affects virus replication and infectivity *in vivo*, we tested a deletion mutant cAvm1, with a complete HVR deletion in the backbone of avian HEV infectious clone, both *in vitro* and *in vivo*. Capped RNA transcripts for the mutant cAvm1 and wild-type avian HEV infectious cDNA clone were transfected into LHM chicken liver cells to determine the replication-competency *in vitro*. On day 6 post-transfection, avian HEV-specific viral antigen was detected in transfected LMH cells by IFA with anti-avian HEV convalescent serum (Figure 3.4). Intracytoplasmic fluorescent signals were detected in the cells transfected with transcripts from both wild-type avian HEV and avian HEV complete HVR-deletion mutant. Mock transfected cells were negative for viral antigen. Detection of viral antigen in cells transfected with complete HVR-deletion mutant cAvm1 indicated that the mutant is replication competent in LMH cells.

The ability of the avian HEV complete HVR-deletion mutant (cAvm1) to replicate and infect chickens were tested by intrahepatic inoculation of SPF chickens via a laparoscopic surgical procedure with capped RNA transcripts from the mutant. Avian HEV-specific RNA was detected variably in fecal and sera samples of the chickens inoculated with wild-type HEV. Six out of the ten chickens in the wild-type avian HEV-inoculated group seroconverted to IgG anti-avian HEV antibodies (Figure 3.5). However, none of the chickens in the mutant avian HEV-inoculated group had detectable avian HEV RNA in the feces or sera, and the chickens remained seronegative throughout the course of study (Table 3.3). Negative-control chickens remained negative for avian HEV infection during the entire course of the experiment. The

results from the chicken study showed that the complete HVR-deletion mutant of avian HEV is non-infectious *in vivo*.

DISCUSSION

The present work deals with the role of HVR in the ORF1 of HEV in viral replication. Recently, we demonstrated that deletions in the HVR can be tolerated by HEV for its infectivity both *in vitro* and *in vivo*, suggesting structural flexibility of the HVR. However, the effects of deletions in the HVR on the replication of HEV remain unknown. Therefore, in this study we aimed to determine the effect of various HVR deletions on HEV replication.

The importance of HVR residues for HEV RNA replication was studied by introducing deletions of various lengths into the subgenomic luciferase replicon pSH-HEV2-luc of genotype 1 human HEV. Replication efficiencies of the RNAs carrying various length deletions in HVR were determined in a transient replication assay by transfecting Huh7 cells with *in vitro*-transcribed replicon RNA. Luciferase activities were determined in cell lysates in triplicate measurements 4 days after transfection and expressed as a percentage relative to that of the parental replicon. The results were normalized for transfection efficiency by using the T7-promoter driven renilla luciferase activity (pRL-TK vector) as an internal control.

Although, the HVR contains amino acid residues dispensable for replication (108), there is no clear demarcation between dispensable and remaining sequences in this region. In this study, we have used a series of deletions mutants: mutants H51-Luc (Δ 711-725), H52-Luc (Δ 711-740) and H53-Luc (Δ 711-750) with N-terminal deletions of the HVR, mutants Hm1-Luc (Δ 729-754), Hm2-Luc (Δ 721-766), and Hm3-Luc (Δ 716-771) with deletions in the central regions of the HVR, mutants H31-Luc(Δ 761-775), H32-Luc (Δ 746-775), and H33-Luc (Δ 736-775) with C-terminal deletions of the HVR. The effect of these deletions on HEV RNA replication *in vitro* was examined. The results showed that small and moderate-sized deletions in the N-terminal region and central region of the HVR were associated with corresponding marked reductions in HEV replication as evidenced by significantly reduced luciferase activity. Larger deletions in these regions resulted in more significant reduction in viral RNA replication. In contrast to the relatively gradual decline in replication levels exhibited by mutants with N-terminal and central region deletions of the HVR, the size of the deletions in the C-terminal region of HVR apparently had little effect on viral RNA replication in Huh-7 cells, as there was

no significant difference in the luciferase activities expressed by mutants H31-Luc (62%), H32-Luc (44%), and H33-Luc (53%) respectively. These results indicate that all the HVR-deletion mutants were replication competent *in vitro*, although deletions in the HVR reduced the viral RNA synthesis in Huh7 cells. The data also indicate that N-terminus and central region of the HVR may contribute more than the C-terminus region of HVR for efficient replication of HEV.

To verify the results obtained with a mammalian strain of HEV, we further examined the effect of HVR deletions on viral replication using a non-mammalian and distant member of the family, avian HEV. Three HVR deletion-mutants of avian HEV: Avm1-luc (Δ 557-603), Avm2-luc (Δ 566-595), and Avm3-luc (Δ 573-587) were constructed using the backbone of avian HEV luciferase replicon, Av-HEV-luc. Following transfection in LMH cells, the luciferase activities were determined in cell lysates in triplicate measurement after 5 days. Compared with the wild-type avian HEV, the viral RNA replication levels of the HVR deletion mutants were considerably reduced. Progressively larger deletions resulted in corresponding decreases in replication levels of avian HEV RNA.

Although the ORF1 HVR (aa707 to 775) contains residues that are dispensable for viral replication as demonstrated previously (108), the association of various length deletions of HVR with different reduced levels of viral RNA replication suggests a possible role of HVR in HEV replication. Replication of the genomes of well-characterized positive-strand RNA viruses, including plant, animal, and insect viruses, involve the establishment of specific interactions between host and viral proteins. Relatively lower levels of viral replication observed for HVR deletion-mutants when compared to wild-type HEV suggest that HVR may play a role in viral RNA replication perhaps via specific interactions with viral and/or host-specific factors in the intracellular environment of infected cells which could modulate the efficiency of viral RNA replication.

Control of structural flexibility is essential for the proper functioning of a larger number of proteins and multiprotein complexes. The nature of interdomain linkages formed by oligopeptides influences the presence and absence of such flexibility, with implications on the protein architecture and function of multidomain proteins (148). It was previously demonstrated that the HVR of HEV overlaps with the proline-rich hinge region of ORF1 (73, 108). Proline is the most common terminal linker residue located between functionally relevant regions of protein structures. Such polypeptides have been termed “molecular rulers” which are necessary

to keep the interactions of other residues in register (22, 148). Control of peptide flexibility through molecular rulers is not only an engineering enterprise, functionally active molecular rulers are also found in nature (105). We speculate that deletions introduced into such linkers like the HVR of HEV may alter specific interaction with viral and/or host factors and modulate the efficiency of viral replication. This may also be a reason for the observed replication gradient among HVR deletion mutants of various sizes, and a larger deletion could interfere with the interaction of domains with their substrates exerting a size limit to maintain correct folding of the functional domain. Deletions at the C-terminal region of HVR did not show a typical replication gradient shown by N-terminal and central region deletions, suggesting that the deletions in the C-terminal region of the HVR likely did not interfere with the folding of HVR domain and/or polyprotein.

To evaluate the effect of the complete HVR deletion on avian HEV infectivity, we first generated a complete HVR deletion mutant of avian HEV (aa Δ 557-603) using the avian HEV infectious cDNA clone as the backbone. The viability of the complete HVR deletion mutant was then verified in LMH cells by IFA six days posttransfection with the RNA transcripts. Detection of avian HEV-specific antigen by IFA confirmed the replication-competency of the complete HVR deletion mutant. To analyze the impact of the complete HVR deletion on the infectivity of avian HEV *in vivo*, 4-week old SPF-chickens were intrahepatically inoculated with capped RNA transcripts generated from the complete HVR deletion mutant. The results showed that the complete HVR-deletion mutant of avian HEV is non-infectious during the 5 weeks period of the study as evidenced by the lack of fecal virus shedding, viremia or seroconversion in the complete HVR-deletion mutant inoculated chickens, though the chickens inoculated with the capped RNA from the wild-type avian HEV became infected. We have previously shown that chickens inoculated with the RNA transcripts from a larger HVR-deletion mutant (aa 557-641) had no viremia or fecal virus shedding, and two of the three chickens were seronegative whereas the third inoculated chicken had a borderline OD value (108). Therefore, the results from this study with a more defined complete HVR deletion further confirm our previous study.

In summary, our results demonstrate significant structural and functional flexibility in the amino acid residues of HVR of HEV and absence of critical functions for a majority of charged residues. However, the results also demonstrate the presence of important amino acid residues in the N-terminal and central regions of the HVR that are important for efficient HEV RNA

replication. Moreover, together with our observation of the loss of infectivity of avian HEV lacking the complete HVR *in vivo*, suggests that the host factors, HVR and HEV replication are intimately connected. Clearly, future studies are needed to determine how the absence or change in particular amino acid residues influence the replication and/or infectivity of HEV, which is beyond the scope of the present study. The structural and functional flexibility of HVR identified in this study indicates that the HVR may be a target for potential attenuation of HEV for the development of a live attenuated vaccine.

ACKNOWLEDGEMENT

This study was supported in part by grants from the National Institutes of Health (AI074667, and AI050611). We thank Pete Jobst and the animal care staff for their assistance in the animal study.

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Figure 3.1

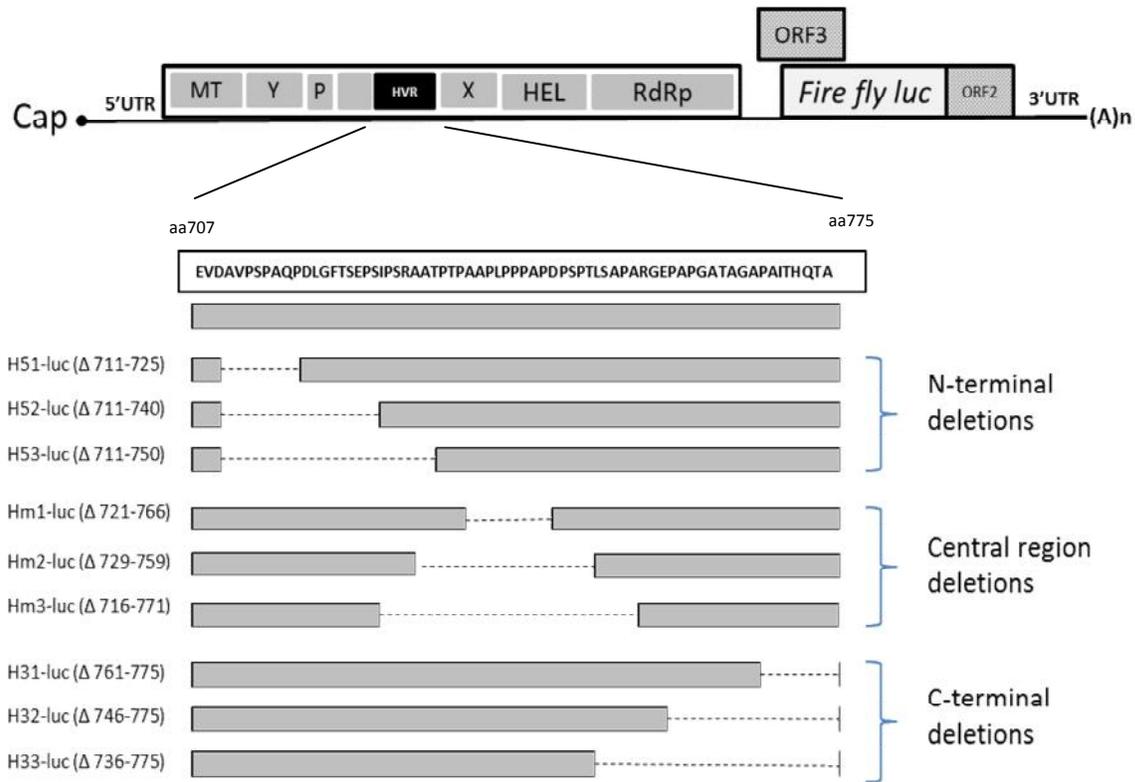


Figure 3.1. Schematic diagrams of the organization of subgenomic HEV luciferase replicon and construction of various HVR deletion-mutants. The putative functional domains: MET, methyltransferase; Y, Y-domain; P, papain-like cysteine protease; HEL, helicase; and RdRp, RNA-dependent RNA polymerase are marked by gray boxes, while the HVR (aa707-775) is boxed in dark. In-frame deletions of the HVR were generated by fusion PCR. The deleted amino acids are shown as a dashed line, and the relative positions are indicated in the individual mutant name.

Figure 3.2

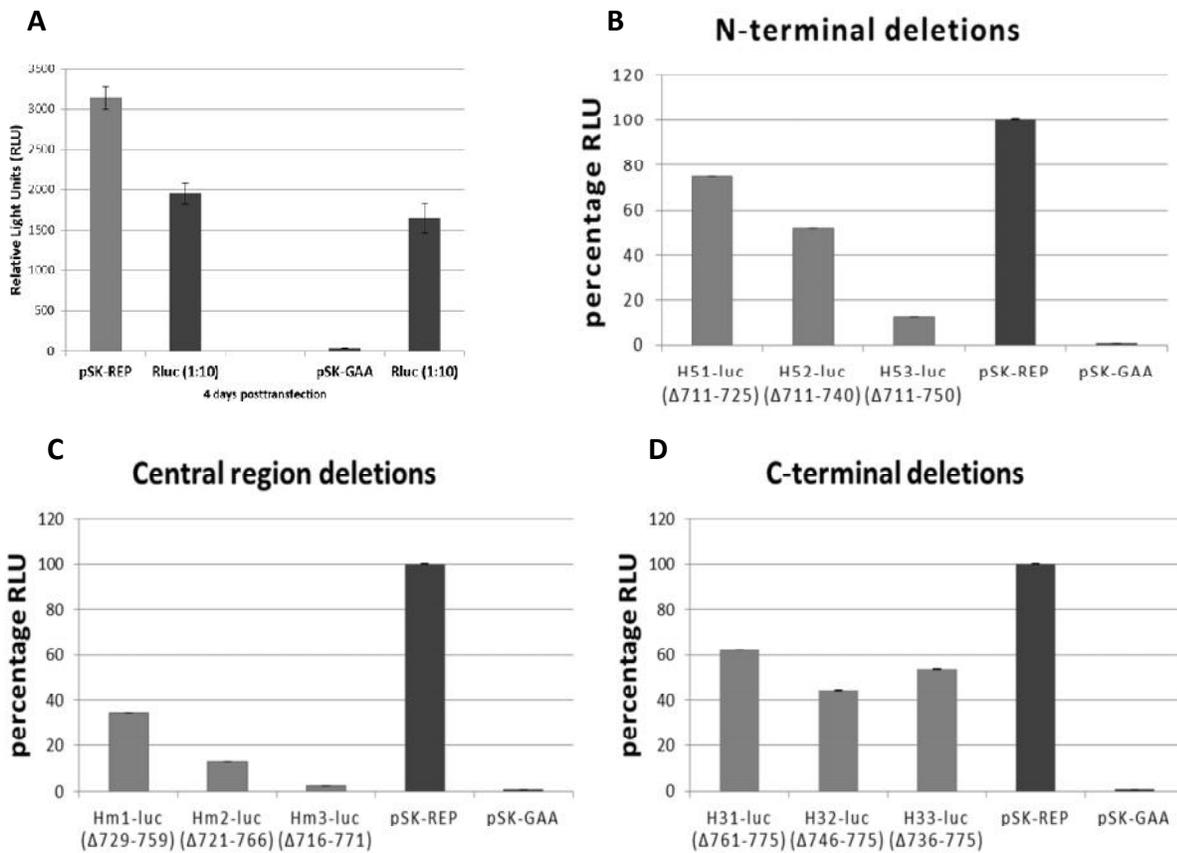


Figure 3.2. Luciferase activity in Huh7 cells transfected with the capped RNA transcripts from genotype 1 human HEV luciferase replicon and its mutant derivatives. Luciferase activity following transfection reflects the replication levels of viral RNAs. Viral RNA replication was measured by determining the firefly luciferase activity in relative light units (RLU) at 4 days post-transfection and normalization for transfection efficiency by using the renilla luciferase activity measured at the same time. The value determined with the parental replicon (pSK-REP) was set as 100% and used as a reference to normalize the replication of all other mutant replicons. The replicon carrying an inactivating mutation (GAA) in the RNA-dependent-RNA-polymerase (RdRp) served as a negative control. Values are means and standard deviations from at least two independent experiments, each measured at least in triplicate. (A) Luciferase activity determined for the parental replicon (pSK-REP), internal control, and the null mutant (pSK-GAA). (B) Relative replication levels of mutants carrying deletions in the N-terminus of HVR. (C) Relative replication levels of mutants carrying deletions within the central region of the HVR. (D) Relative replication levels of mutants carrying deletions within the C-terminus of the HVR.

Figure 3.3

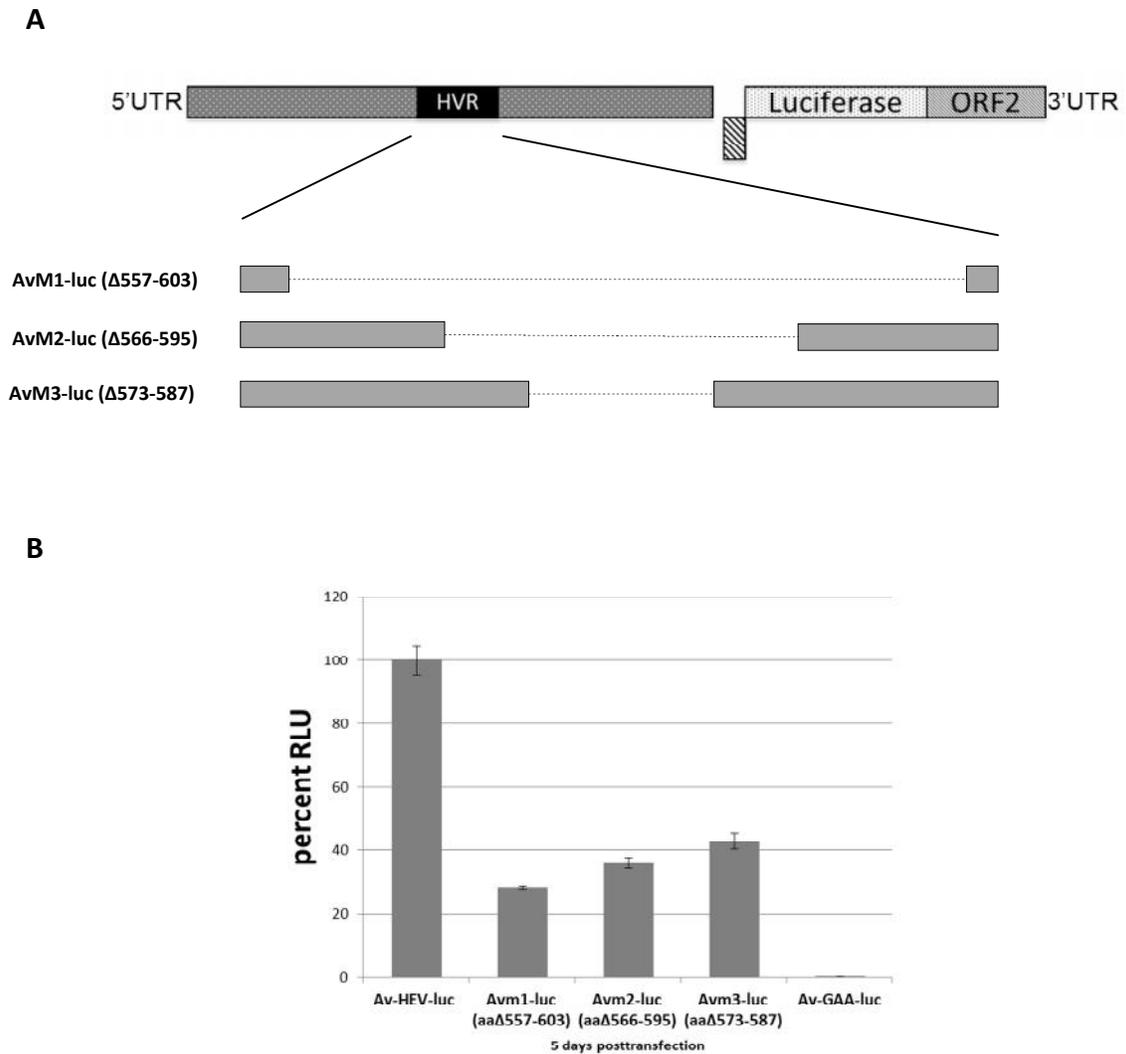


Figure 3.3. Schematic diagram of the organization of subgenomic luciferase replicon of avian HEV, construction of HVR deletion-mutants, and relative replication levels of replicon RNAs in LMH cells. (A) Organization of subgenomic luciferase replicon of avian HEV and construction of HVR deletion-mutants. In-frame deletions of the HVR (aa557-603) were generated by fusion PCR. The deleted amino acids are shown as a dashed line, and the relative positions are indicated in the individual mutant name. (B) Relative replication levels of parental replicon (Av-HEV-Luc) and HVR-deletion mutants 5 days posttransfection LMH cells.

Figure 3.4

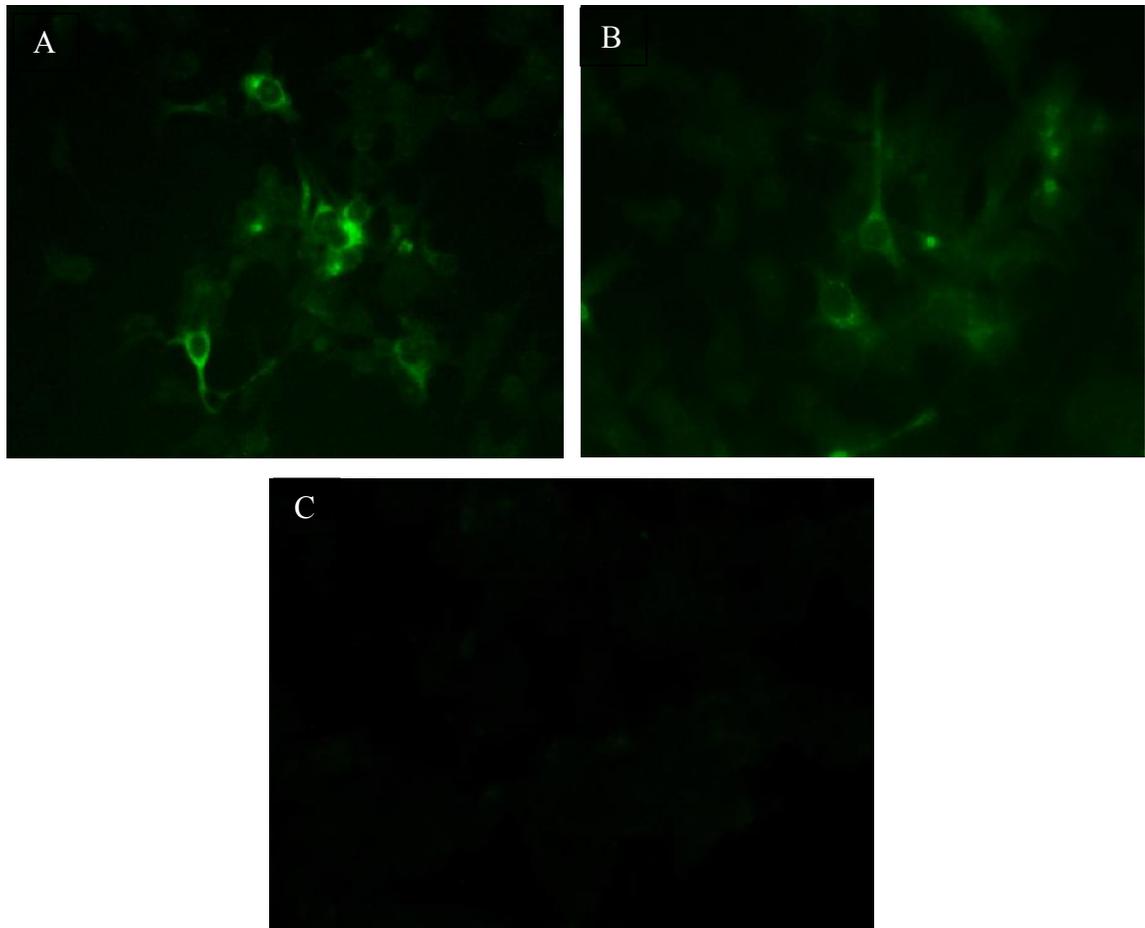


Figure 3.4. IFA of LMH chicken liver cells following transfection with capped RNA transcripts. Immunofluorescence microscopy of LMH chicken liver cells following 6 days of transfection with capped RNA transcripts from the wild-type avian HEV (A), complete HVR deletion-mutant of avian HEV cAvm1 (B), and mock-transfected cells (C).

Figure 3.5

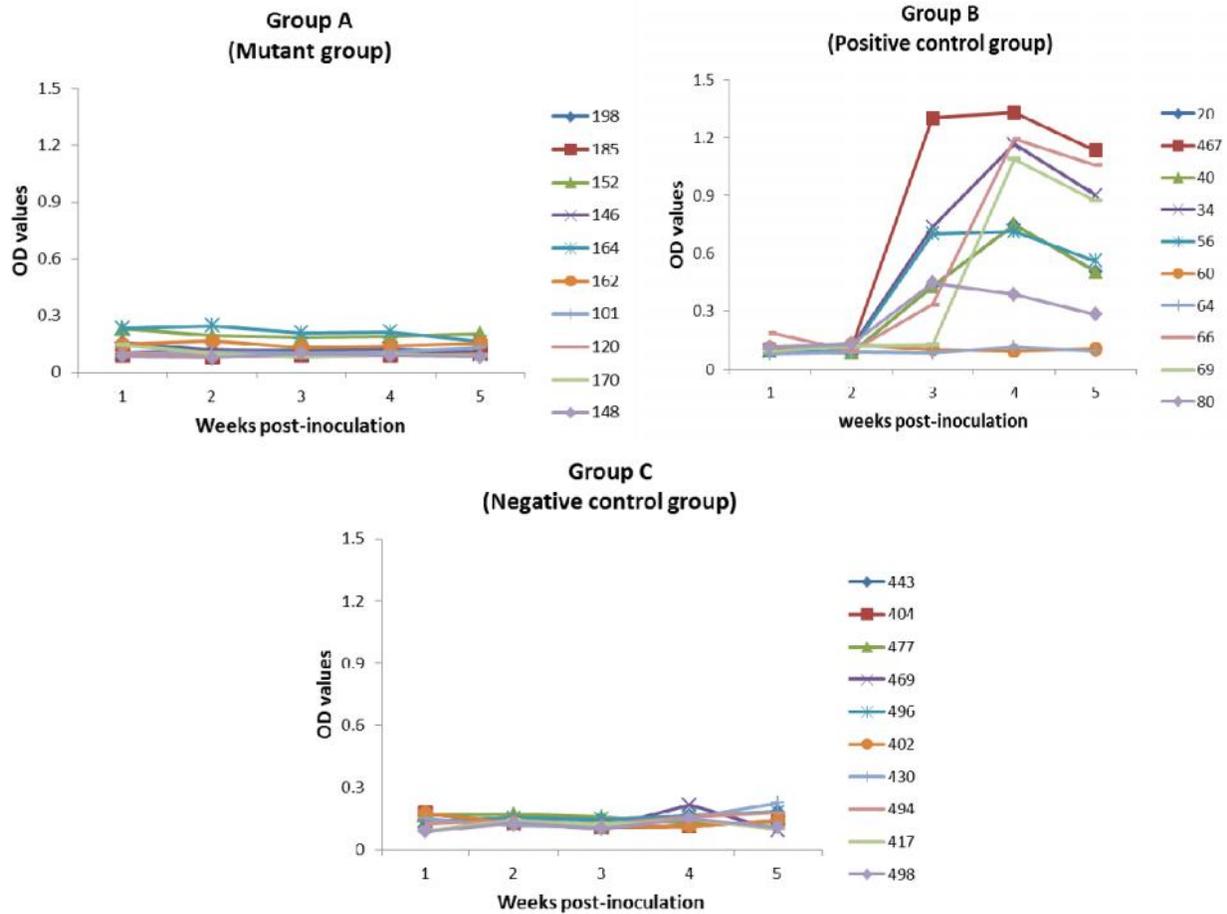


Figure 3.5. Seroconversion to IgG anti-HEV in chickens inoculated with capped RNA transcripts from the wild-type avian HEV infectious clone and the complete HVR deletion mutant of avian HEV. IgG anti-HEV was plotted as the ELISA optical density (OD) (A_{405}), and the ELISA cutoff value was 0.30. Chickens 198, 185, 152, 146, 164, 162, 101, 120, 170, and 148 were each inoculated with RNA transcripts from HVR deletion mutant cAVm1 (group A). Chickens 20, 467, 40, 34, 56, 60, 64, 66, 69, and 80 (group B) were each inoculated with RNA transcripts from the wild-type avian HEV infectious cDNA clone as positive controls, and chickens 443, 404, 477, 469, 496, 402, 430, 494, 417, and 498 (group C) were intrahepatically inoculated with PBS buffer as negative controls.

Table 3.1. Primers used for generation of genotype 1 human HEV plasmid constructs

Primer	Sequence ^a (5' →3')	Polarity	Purpose
HuLP1	GATTGGCATGCTACAGGCTGTTGCTGATGG	Forward	PCR
HuLP2	GCGAATTCCTATTATTCTTGCCGCCCTTCTTGG	Reverse	PCR
HuLP3	TTTGGCGTCTTCCATGGTCGCGAACCCATG	Reverse	PCR
HuLP4	CATGGGTTTCGCGACCATGGAAGATGCCAAAAAC	Forward	PCR
HuGAAF	GCTGCCTTTAAAGGTGCTGCTTCGATAGTGCT	Forward	Codon mutagenesis
HuGAAR	AGCACTATCGAAGCAGCACCTTTAAAGGCAGC	Reverse	Codon mutagenesis
HuF	GGGAGCATGCTCAGAAGTTTATAACACGCC	Forward	PCR
HuR	GTACCTCTGGTAAAATGCATGACAGAGCCC	Reverse	PCR
Hur	AGC ATC AAC CTC CGA CCA AGT GCG GGT GTA	Reverse	PCR
Huf1	TCG GAG GTT GAT GCT TCT ATA CCT AGT AGG	Forward	PCR
Huf2	TCG GAG GTT GAT GCT CCC CCC CCT GCA CCG	Forward	PCR
Huf3	TCG GAG GTT GAT GCT CTC TCT GCT CCG GCG	Forward	PCR
Huf4	GAG CCT TCT ATA CCT GCT CCT GGC GCT ACC	Forward	PCR
Huf5	CAG CCC GAC TTA GGT GCC CCA GCC ATA ACC	Forward	PCR
Huf6	GTT CCT AGT CCA GCC CAC CAG ACG GCC CGG	Forward	PCR
Hur1	AGG TAT AGA AGG CTC AGA TGT AAA ACC TAA	Reverse	PCR
Hur2	ACC TAA GTC GGG CTG GGC TGG ACT AGG AAC	Reverse	PCR
Hur3	GGC TGG ACT AGG AAC AGC ATC AAC CTC CGA	Reverse	PCR
Huf7	GAG CCG GCT CCT GGC CGC CGC CTG CTC TTT	Forward	PCR
Huf8	CCT GCA CCG GAT CCT CGC CGC CTG CTC TTT	Forward	PCR
Huf9	ACA CCT ACC CCG GCG CGC CGC CTG CTC TTT	Forward	PCR
Hur4	GCC AGG AGC CGG CTC ACC ACG CGC CGG AGC	Reverse	PCR
Hur5	AGG ATC CGG TGC AGG GGG GGG TAG AGG GGC	Reverse	PCR
Hur6	CGC CGG GGT AGG TGT GGC GGC CCT ACT AGG	Reverse	PCR

^a Underlined nucleotides were required for cloning or mutagenesis purposes.

Table 3.2. Primers used for generation of avian HEV plasmid constructs

Primer	Sequence ^a (5' →3')	Polarity	Purpose
Av-p1A	<u>CCCGTTAACA</u> AATATGCCCTTGCCG	Forward	Deletion mutagenesis
Av-p1F	CCAGGCGCCCCGTTACCAATATGCCCTTGCC	Forward	PCR
Av-p1R	GGTCCGCGGGGCAATGGTTATACGGGAACGG	Reverse	PCR
Av-p2A	AGCGGAGACGGCCGGTGGTGCAGTT	Forward	PCR
Av-p2B	CCGGCCGTCTCCGCTGGCTTGA ^c CTCCGACGCGC	Reverse	PCR
Av-p3A	CGGGGGTGCCGGTACAGGGCCAGCGGA	Forward	PCR
Av-p3B	GTACCGGCACCCCCGCCTGAGGTCAGTGAGTCC	Reverse	PCR
AvFF	GGTACGAAGTCTGCAGTTAGCAAGTGG	Forward	PCR
AvR	CGGG <u>TTAACA</u> AGCCAGTCGGCGGCA	Reverse	PCR
AvEcoRVF	GGGG <u>GATATC</u> TGCGTGGCCGAAAAC ^c TTTG	Forward	PCR
AvSalIR	GCGG <u>TCTGACT</u> TATTACACGGCGATCTTG	Reverse	PCR
AvBsmBif	<u>CGTCTCAAT</u> GGAAGATGCCAAAAACA	Forward	PCR
AvBsmBlr	<u>CGTCTCACCA</u> TCCCACCCCACTTTCCT	Reverse	PCR
AvGAAF	GTTGTGTTTCAAAGGTG <u>CTGCT</u> AGTGTCGTTGTCTGTG	Forward	Codon mutagenesis
AvGAAR	CACAGACAACGACACTAG <u>CAGCAC</u> CTTTGAAACACAAC	Reverse	Codon mutagenesis

^a Underlined nucleotides were required for cloning or mutagenesis purposes

Table 3.3. Detection of avian HEV RNA in fecal, and serum samples of SPF chickens intrahepatically injected with RNA transcripts from the wild-type avian HEV infectious clone and the complete HVR deletion mutant of avian HEV

Group	Sample	No. positive / total no tested at weeks-postinoculation				
		1	2	3	4	5
Group A (Mutant group)	Feces	0/10	0/10	0/10	0/10	0/10
	Serum	0/10	0/10	0/10	0/10	0/10
Group B (Pos. control group)	Feces	0/10	5/10	8/10	6/10	5/10
	Serum	0/10	0/10	5/10	4/10	3/10
Group C (Neg. control group)	Feces	0/10	0/10	0/10	0/10	0/10
	Serum	0/10	0/10	0/10	0/10	0/10

Chapter 4

The hypervariable region (HVR) of hepatitis E virus is not genotype-specific and can be functionally exchanged for *in vitro* virus replication

R. S. Pudupakam, Laura Cordoba, Y. W. Huang, and X. J. Meng

ABSTRACT

The proline-rich hinge region of the open reading frame 1 (ORF1) encoding non-structural polyprotein overlaps with the most variable part of the hepatitis E virus (HEV) genome, the hypervariable region (HVR). The HVR contains sequences that are dispensable for viral infectivity. We have previously demonstrated that the HVR contains critical amino acid residues that modulate the replication efficiency of HEV. In this present study, we constructed three chimeric viruses with swapped HVR from different genotypes: chimeras pSKHEV2-Sw and pSKHEV2-Av with the HVR from genotype 3 swine HEV (aa707-790) and the avian HEV (aa557-603) replacing that of the genotype 1 human HEV (aa707-775), respectively, in the backbone of genotype 1 human HEV, and chimera pSHEV3-Hu with the HVR from genotype 1 HEV replacing that of the genotype 3 swine HEV in the backbone of genotype 3 HEV. Capped RNA transcripts from these chimeras and wild-type HEV were transfected into Huh7 cells to evaluate replication competency. HEV-specific viral antigen was detected by an immunofluorescence assay (IFA) in Huh7 cells transfected with the RNA transcripts of all three chimeras as well as the wild-type HEV, indicating that these chimeric viruses with swapped HVR are replication competent. Cell lysates of Huh7 cells transfected with the RNA transcripts from each chimera were used to infect HepG2 cells. Infectious HEV particles were detected in the HepG2 cells inoculated with the pSKHEV2-Sw and pSHEV3-Hu cell lysates but not with pSKHEV2-Av lysate. The results from this study demonstrated that exchanging the HVR from different genotypes of mammalian HEV do not abolish the virus replication-competency or infectivity, indicating that HVR is not genotype-specific for virus replication. The results also showed that the HVR of avian HEV appears to be non-compatible with the mammalian HEV infectivity, further supporting the classification of avian HEV as a separate genus.

INTRODUCTION

Hepatitis E virus (HEV) is a positive-strand RNA virus belonging to the family *Hepeviridae* (7, 18). The viral genome is approximately 7.2 kb in length and is composed of three open reading frames (ORFs 1-3) flanked by 5' and 3' non-translated regions (5' and 3' NTRs) (12, 22). The largest ORF1, which encompasses the 5' proximal two-thirds of the genome, is believed to encode the protein functions required for HEV RNA synthesis (15, 17, 19). The two overlapping open reading frames, ORF2 and ORF3, encode for the major capsid protein and a small multifunctional phosphoprotein, respectively, from the same bicistronic sub-genomic RNA (9, 10, 26).

The putative domains needed for viral genome replication are located in a sequential order from the N to the C termini of the ORF1 polyprotein, as methyltransferase, protease, helicase, and RNA-dependent RNA polymerase, respectively (1, 13-15, 17, 19). The non-structural polyprotein of HEV also contains a hypervariable region (HVR) that overlaps with the proline-rich sequence (PRS) that is located between the N-terminus of the X domain and the C-terminal portion of putative papain-like protease domain (15). A "proline hinge" region was also recognized in closely related rubella virus (15, 25). The HVR varies both in length and in sequence among different HEV strains.

The inefficient replication of HEV in cell culture systems has slowed down progress in understanding the biology and life cycle of HEV. The problem has been partially overcome either by characterizing individually expressed proteins from plasmid vectors or by transfecting cells and intrahepatically inoculating animals with the RNA transcripts generated from infectious cDNA clones and their derived mutants (2, 6, 11, 19, 20, 23, 24). Recently, an *in vitro* system allowing the study of HEV infectivity in a cell culture has been reported, and the infectious virions produced in cell culture appeared to be identical to those of virions produced *in vivo* (5).

The role of the HVR of HEV in viral protein function has not been determined. It has been demonstrated that HEV can tolerate partial deletions in HVR for its infectivity (19). In addition, the HVR has been shown to be capable of influencing the replication efficiency of HEV, either by direct contact with viral and/or host factors or due to a more indirect effect on the structure of neighboring functional domains (R. S. Pudupakam et al., unpublished data).

In this study, we wished to study in more detail the role of the HVR in HEV life replication was further investigated in more detail. As the HVR exhibits extensive sequence

variations among HEV strains from different genotypes, we initially examined the interchangeability of HVRs and demonstrated that, for virus replication *in vitro*, the sequences contained in HVR are not genotype-specific and could be substituted between genotypes for virus replication, despite the observed significant differences in the length and sequence of the HVR. Furthermore, we demonstrated that HEV genomes with swapped HVRs from different genotypes could support production of infectious HEV particles *in vitro*.

MATERIALS AND METHODS

Cell lines and infectious cDNA clones: A sub-clone of Huh7 cell line, S10-3(5, 10), which is a relatively more permissive cell line for HEV replication, and the genotype 1 human HEV (Sar55 strain) infectious clone(8), were gifts from Drs. Suzanne Emerson and Robert Purcell at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH. The HepG2/C3A (CRL-10741) cells were purchased from the American Type Culture Collection (Manassas, VA). Cell monolayers were grown in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen), 100 U penicillin ml⁻¹, 10 µg streptomycin ml⁻¹, at 37 °C in a humidified atmosphere of 5% CO₂ in 37 °C. The infectious cDNA clone of the genotype 3 HEV used in this study was reported previously (11).

Construction of chimeric hepatitis E viruses with swapped HVR between different genotypes: Infectious cDNA clones of genotype 1 human HEV (pSK-HEV2) and genotype 3 swine HEV (pSHEV3) were used to construct HEV chimeras, using standard mutagenesis techniques. Primers used to construct the chimeras are shown in Table 4.1. Amino acids 707-775 (nt 2119 to 2325) in the ORF1 of genotype 1 human HEV were replaced with aa707-790 (nt 2119 to 2370) of the genotype 3 swine HEV to construct the chimeric mutant pSKHEV2-Sw. Forward and reverse fragments of human HEV were amplified using the primer sets HuF/Hu r, and Huf/HuR, respectively. The genotype 3 swine HEV HVR fragment that has to be inserted was amplified with the primers Swf/Swr. The *BsmBI* restriction enzyme site that was engineered into primers Hur, Huf, Swr, and Swf were used to ligate the three fragments generated. Anchor primers HuF and HuR containing restriction enzyme sites *Sph I* and *Nsi I*, respectively, were

used to amplify the final ligation product, which was then ligated into the backbone of genotype 1 human HEV digested with the same enzymes.

Amino acids 707-775 (nt 2119 to 2325) in the ORF1 of genotype 1 human HEV were replaced with aa 557 to 603 (nt 1669 to 1809) of the avian HEV to construct the chimeric mutant pSKHEV2-Av. Forward and reverse fragments of the genotype 1 human HEV were amplified using the primer sets HuF/Hu r, and Huf/HuR, respectively. The HVR of avian HEV that had to be inserted was amplified from the infectious cDNA clone of avian HEV strain (pT7-aHEV) with the primers Av f/Av r, and was subsequently cloned into the backbone of the genotype 1 human HEV using a similar strategy as described above.

Chimeric mutant pSHEV3-Hu was constructed by replacing aa 707-790 corresponding to nucleotides (nt) 2119 to 2370 in the ORF1 of the genotype 3 swine HEV with aa 707-775 (nt 2119 to 2325) of the genotype 1 human HEV. Restriction site *Mlu I* was engineered flanking the HVR of the genotype 3 swine HEV by site-directed mutagenesis using the primers SwSMF and SwSMR. The HVR of human HEV was amplified with the primers HuHVrf and HuHVRr containing the restriction site *Mlu I*, and then ligated into the backbone of the genotype 3 swine HEV using the same restriction sites.

***In vitro* transcription and transfection:** The two chimeric virus constructs (pSKHEV2-Sw, and pSKHEV2-Av) with the backbone of genotype 1 human HEV cDNA clone were linearized using a *Bgl II* restriction site directly downstream of the engineered poly(A) sequence. The chimeric virus construct (pSHEV3-Hu) with the genomic backbone of the genotype 3 swine HEV was linearized using a *Xba I*, followed by phenol-chloroform extraction and ethanol precipitation. The RNA transcripts were produced using the mMessage mMachine T7 Kit (Ambion) according to the manufacturer's instructions. For synthesis of 5'-capped RNA transcripts, 1 µg of linearized plasmid was transcribed at 37°C in a 20-µl reaction mixture containing 2 µl 10X reaction buffers, 10 µl 2X nucleoside triphosphate/Cap, 2 µl enzyme mixtures, and an additional 1 µl 30 mM GTP stock for capping. The integrity of transcripts was analyzed by electrophoresis in 1% agarose gels. The yields of the *in vitro*-generated RNA transcripts were determined using the Quant-iT RiboGreen RNA assay Kit (Molecular Probes) according to the manufacturer's instructions.

For the transfection of Huh7 cells with RNA transcripts, 10 µgs of the transcribed RNA from each of the chimeras as well as the wild-type HEV were mixed with 10 µl of DMRIE-C transfection reagent (Invitrogen) and 1 mL of Opti-MEM (Invitrogen), and then added onto

Huh7 cells seeded at 60 % confluence in a T-25 flask. After 5 hours of incubation at 34.5°C, the transfection mixture was replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and the plates were again incubated at 34.5°C.

Immunofluorescence assay (IFA) and confocal microscopy: Three days post-transfection, the Huh7 cells were trypsinized and replated onto wells of Labtek chamber slides. On day 6, the Huh7 cells were rinsed with PBS, fixed with acetone at room temperature for 10 minutes, and air-dried. A 1: 200-diluted anti-HEV convalescent serum from a chimpanzee experimentally infected with a genotype 1 human HEV was added to the fixed cells and incubated for 45 minutes at room temperature. After washing with cold PBS (1X), 1 : 2000-diluted Alexa Fluor 488-conjugated goat anti-human immunoglobulin G (Molecular Probes) was added and incubated at room temperature for 30 min. Vectashield (Vector Laboratories) mounting medium was added to the washed wells, and viewed under a Zen3000 confocal laser (ZEISS).

Preparation of cell lysates for infectivity assay: Cell lysates were prepared as described previously(5). Confluent monolayers of Huh7 cells in a T25 flask were trypsinized at 9 days post-transfection and centrifuged for 1.5 min at 800 rpm followed by 1.0 min at 13,200 rpm. Supernatant was aspirated, and the cell pellet was stored at -80°C. Frozen pellets were lysed at room temperature by adding 0.9 ml water per cell pellet from a T25 flask and vortexed vigorously until the pellet is dispersed in the solution. The sample was again vortexed 1 to 2 times within 10 min, followed by addition of 0.1 ml of 10x concentrated PBS, and removal of debris by centrifugation at 13,200 rpm for 2 minutes.

Infectivity assay: Confluent monolayers of HepG2 cells were trypsinized and diluted 4 times in growth medium, and 0.1 ml of the cells was carefully added to 0.4 ml of growth medium in a well of an eight-well glass chamber slide. After 1 to 2 days of incubation at 37°C, a sparse population of monolayers was observed. The medium in the wells of the chamber slide was then replaced with duplicate 100- μ l samples of the cell lysates (100 μ l undiluted lysate and 100 μ l 10-time diluted lysates in 10% DMEM). The cells were allowed to incubate at 34.5°C in a 5% CO₂ atmosphere for 1 hour, and then the cell lysate was aspirated and replaced with 0.4 ml of growth medium containing antibiotics and 2% of DMSO. After 5 to 6 days of incubation at 34.5°C, the cells were fixed and stained by IFA as described previously(5).

RESULTS

Inter-genotypic chimeric viruses with swapped HVR's are replication-competent *in vitro*:

Extensive sequence variability within the HVR of mammalian HEVs and avian HEV was reported previously (19). The presence of critical amino acid residues in HVR that may modulate the replication efficiency of HEV has been suggested (R. S. Pudupakam., unpublished data). In this study, we sought to determine if the HVR's contain important sequences that are genotype-specific for virus replication. Three intergenotypic chimeric viruses with swapped HVRs were constructed: chimeras pSKHEV2-Sw with the genotype 3 HVR replacing that of genotype 1 HEV, pSHEV3-Hu with genotype 1 HVR replacing that of the genotype 3 swine HEV, and pSKHEV2-Av with the avian HEV HVR replacing that of the genotype 1 human HEV.

To examine the replication competency of the chimeric viruses, a sub-clone of Huh7 cells (S10-3) was transfected with capped RNA transcripts from pSKHEV2-Sw, pSHEV3-Hu, and pSKHEV2-Av, respectively, along with the RNA transcripts from the wild-type HEVs, pSKHEV2 and pSHEV3, which were used as a positive control in all experiments in this study. Cells were stained for the presence of the ORF2 protein by IFA on day 5 post-transfection. ORF2-specific viral antigens were detected in transfected Huh7 cells with anti-HEV convalescent serum from a chimpanzee. Cytoplasmic fluorescent signals were detected in the cells transfected with RNA transcripts from both wild-type HEVs and the chimeras (Figure 4.1). Mock-transfected cells were negative for viral antigen. Detection of viral antigens in cells transfected with the capped RNAs from the chimeric viruses indicated that the substitution of the HVR's does not affect the replication-competency of HEV and that the HVR sequences are not genotype-specific for *in vitro* virus replication.

Inter-genotypic chimeric viruses with swapped HVRs are capable of producing infectious virus *in vitro*:

Viral RNA replication was not affected by the HVR exchange, but the effects on the steps downstream of RNA replication cannot be ruled out. Therefore, we performed an *in vitro* infectivity assay to determine if the HVR exchange has any effect on the viral particle assembly process. It was previously demonstrated that infectious particles of HEV were produced in Huh7 cells transfected with HEV RNA. The majority of the infectious particles were retained within the cells, indicating cell lysates as the preferred source of infectious particles for

the infectivity study. HepG2 cells were successfully infected with the lysate of Huh7 cells containing HEV particles (4).

To assay for infectious virus production, HepG2 cells were infected with lysates of transfected Huh7 cells collected at 9 days post-transfection. Cells were examined by IFA for the presence of viral antigen. Cytoplasmic fluorescent signals were detected in the cells infected with the lysates of Huh7 cells transfected with the chimeras, pSKHEV2-Sw, pSHEV3-Hu, as well as genotype 1 human (pSKHEV2) and genotype 3 swine (pSHEV3) HEV strains, indicating the presence of infectious particles in the Huh7 cell lysates. However, the HepG2 cells infected with pSKHEV2-Av lysates did not show any detectable viral infection (Figure 4.2).

DISCUSSION

In the present study, we demonstrated that the HVR is functionally interchangeable between different mammalian HEV genotypes with respect to virus replication competency and infectious virus production. We constructed three inter-genotypic HEV chimeras with swapped HVRs to study the effect of the genetic variability in the HVR among different genotypes on the viability and production of infectious virus particles. Capped RNA transcripts from these chimera and wild-type HEVs were transfected into Huh7 cells. Five days post-transfection cells were stained by IFA with anti-ORF2 chimpanzee antibody. The results showed that all chimeric viruses with swapped HVR were able to replicate in Huh7 cells.

We have previously demonstrated that HVR is dispensable for replication and is not absolutely required for infectivity (19), therefore we hypothesized that the HVR exchange should not abolish HEV infectivity either. To test this hypothesis, the cell lysates from Huh7 cells transfected with the RNA transcripts of different chimeras as well as the wild-type viruses were used to infect HepG2 cells. The results showed that the chimeras, pSKHEV2-Sw and pSHEV3-Hu were able to infect HepG2 cells as did the wild-type virus. However, infection was not detected in lysates from cells transfected with the chimera pSKHEV2-Av. The absence of a detectable fluorescent signal in HepG2 cells infected with pSKHEV2-Av could be explained by the presence of very few infectious particles in the cell lysates or the HVR from an avian HEV is not compatible with the mammalian strains of HEV. This observation further supports the proposed classification of avian HEV as a separate genus.

Although chimeric HEV genomes with swapped HVR are viable and supported the production of infectious virions, the virus production was less efficient compared with the corresponding wild-type HEV of the same genotype. Relatively few viral particles of HEV were detected in Huh7 cells transfected with capped RNA transcripts from chimeras. We speculate that the HVR-swap chimeras did not affect the secondary structure and thus preserved the overall genomic structure and folding of the ORF1 functional domains, but the swaps may have disrupted genotype-specific protein-protein interactions. It is possible that, depending on the swapped HVR sequences, viruses with dramatically different biological or pathological properties may arise. It would be interesting to know if HEV strains from different genotypes evolved the HVR sequences to specifically exploit and interact with certain host cellular machineries and contribute to viral pathogenesis

We recently reported that HVR contains amino acid residues critical for efficient replication, and deletion of which will result in marked reduction in viral replication levels (R. S. Pudupakam., unpublished data). Removal of larger or near complete HVRs resulted in attenuation of HEV *in vivo*. These data indicated a synergistic interaction between the HVR domain and viral and/or host sequences in viral infectivity. As we explained earlier, HVR overlaps with the proline-rich hinge region located at the N-terminus of the X-domain in the non-structural ORF1 polyprotein. We have identified the presence of SH3 motifs in the HVR of HEV, including all the four genotypes of mammalian HEVs and avian HEV. Several SH3 adaptor motifs have also been identified in the proline-rich hinge region of the closely related rubella virus (25). Apart from the structural proteins, SH3 motifs have also been identified in the non-structural proteins and dispensable regions of many viral proteins (3, 16, 21). Though these SH3 motifs are not absolutely required for virus replication or infectivity, they play an important role in viral pathogenesis by enhancing their replication efficiency and infectivity (16, 21). HEV may exploit these SH3-mediated interactions to enhance its replication and/or infectivity. Clearly, further studies are warranted to elucidate such potential interactions.

While the results from this study indicate a functional homology between the HVR domains from different genotypes, they also support the idea of the HVR as a peptide linker sequence for proper folding of functional elements required for efficient virus replication and infectivity. Further in-depth structure-function analyses of HVR may be required to definitively

identify potential physiologically relevant interaction sites of HVR, and their viral and/or cellular counterparts.

In summary, the present study demonstrates that the heterologous HVR domain between different HEV genotypes is able to effectively interact with viral and/or host factors to achieve the necessary functions for viral replication and infectivity. The availability of a spectrum of chimeric HEV genomes differing in their HVR is important for comparative analyses of the processes that are governed by the HVR. One obvious application will be the identification of potential virulence determinants and the development of potential modified live-attenuated vaccines.

ACKNOWLEDGEMENT

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

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Figure 4.1

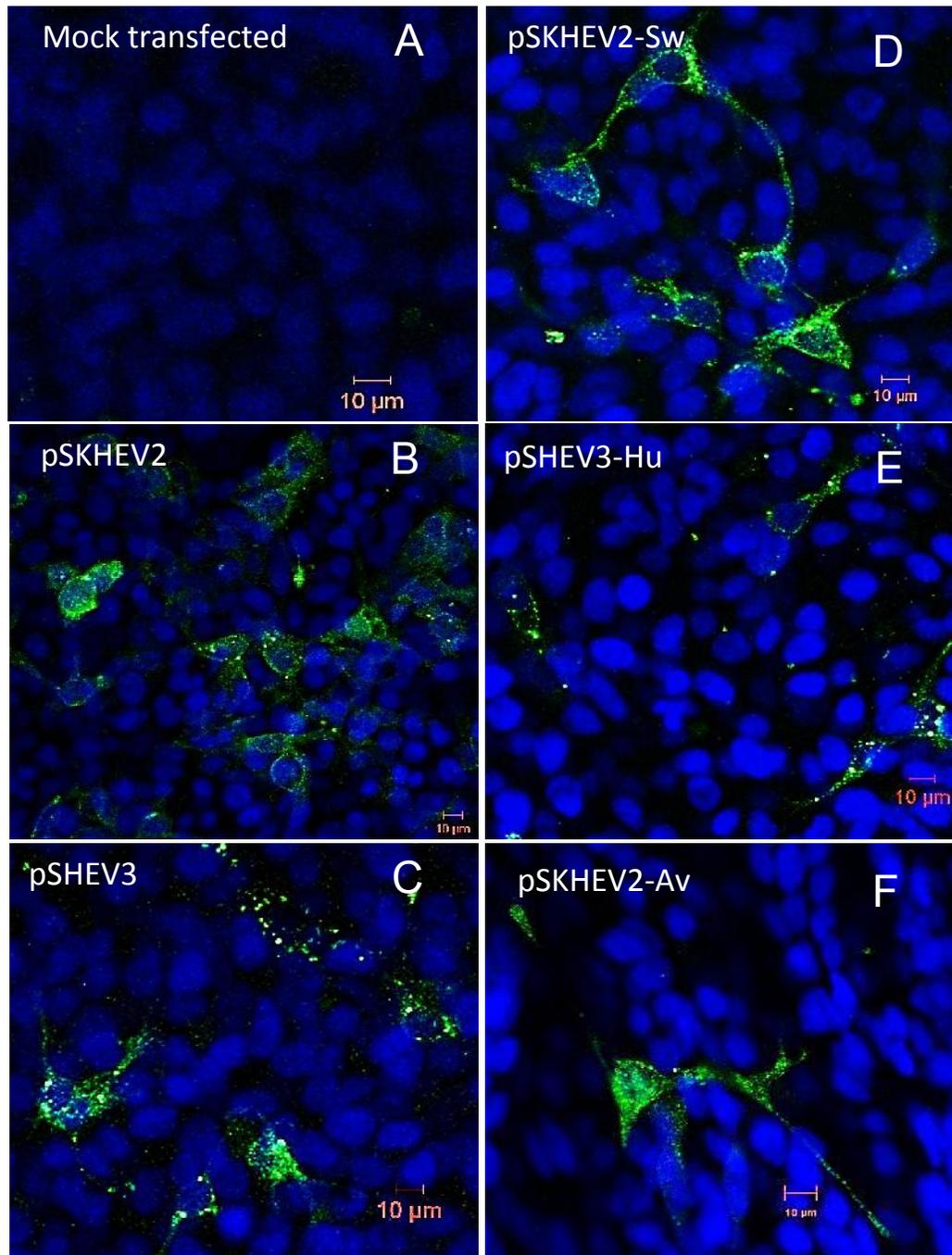


Figure 4.1. Immunofluorescent staining of Huh7 cells transfected with capped RNA transcripts from cDNA clones of the wild type HEVs (pSKHEV2, a genotype 1 HEV and pSHEV3, a genotype 3 swine HEV) and their derivative chimeras (pSKHEV2-Sw, pSKHEV2-Av, and pSHEV3-Hu). At 5 days post-transfection, cells were stained for ORF2 antigen (green) and viewed by confocal microscopy. These images are representative of many cells that were examined.

Figure 4. 2

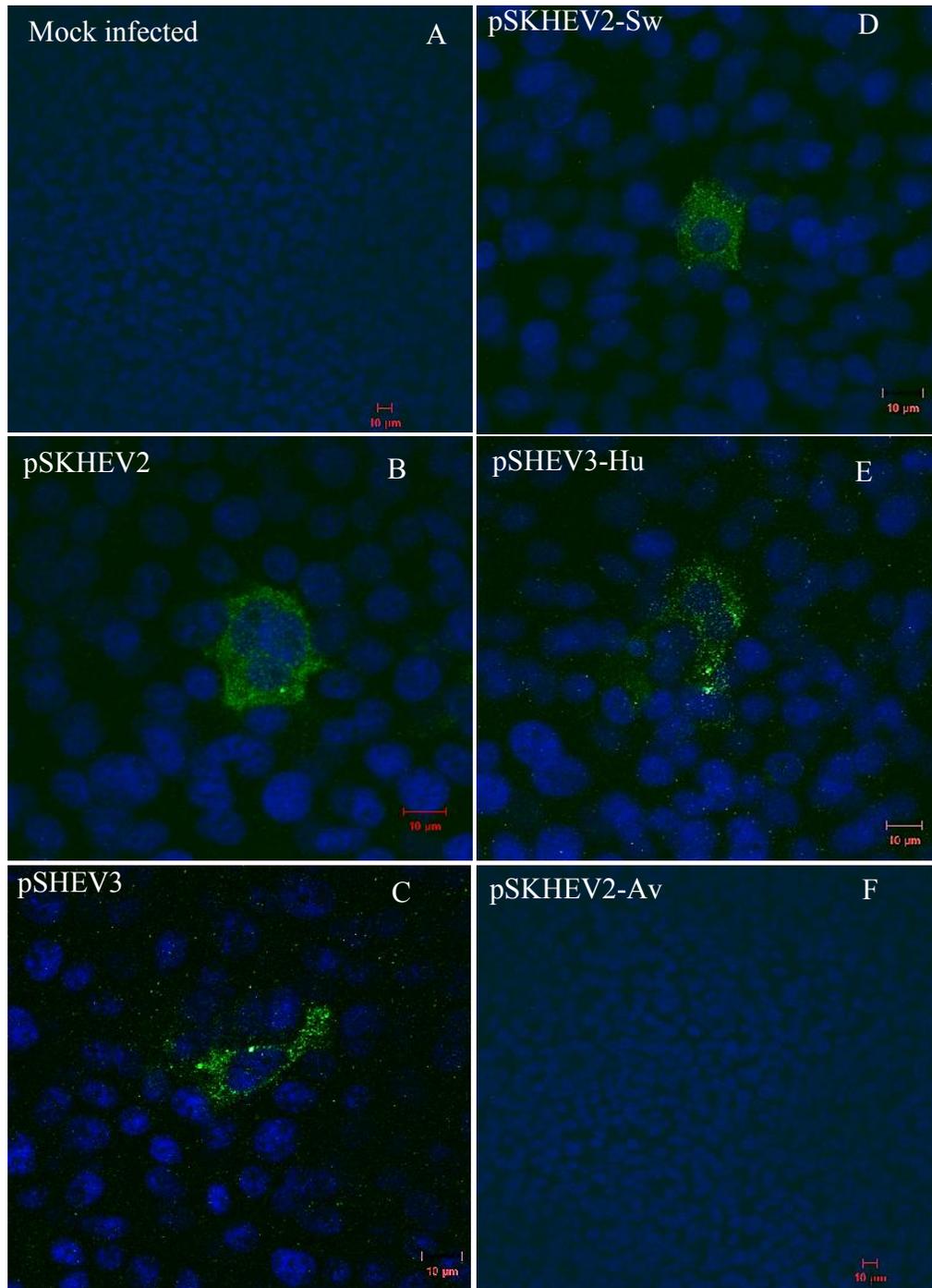


Figure 4.2. Immunofluorescent staining of HepG2 cells infected with lysates of Huh7 cells transfected with RNA transcripts from wildtype and chimeric viruses that were tested positive for the presence of viral antigen after transfection. Cell lysates were collected from cells transfected with wild-type HEVs and its derivative chimerass on day 9 post-transfection. HepG2 cells were stained for ORF2 antigen (green) and viewed by confocal microscopy. These images are representative of many cells that were examined.

Table 4.1. Oligonucleotide primers used for the construction of chimeric hepatitis E viruses with swapped HVR

Primer	Sequence ^a (5' →3')	Polarity	Purpose
HuF	GGGAG <u>CATGCT</u> CAGAAGTTTATAACACGCC	Forward	PCR
HuR	GTACCTCTGGTAAA <u>ATGCAT</u> GACAGAGCCC	Reverse	PCR
Huf	<u>CGTCTCATT</u> CCCGGCATCGCCGCTGCTCTT	Forward	PCR
Hur	<u>CGTCTCACGACCA</u> AGTGCGGGTGTAAGTG	Reverse	PCR
Swf	<u>CGTCTCAGTCGACAT</u> CTGGCTTTTCTAGCG	Forward	PCR
Swr	<u>CGTCTCAGGA</u> AAGCGGGGGTGTTGGTGGC	Reverse	PCR
Avf	<u>CGTCTCAGTCGACT</u> TGGTCAAAGTGCACACCGGCC	Forward	PCR
Avr	<u>CGTCTCAGGA</u> AAGCGAACCTCGCGGTCGGAGTCAA	Reverse	PCR
SwSMF	AGGGGACTTTGTATAC <u>GCGT</u> ACTTGGTCAACATCTG	Forward	Codon mutagenesis
SwSMR	CCCGCCTCCCGCAC <u>GCGT</u> CGTCTCCTCTAC	Forward	Codon mutagenesis
HuHVRf	GGG <u>ACGCGT</u> ACTTGGTCAGAGGTTGATGCTGTTCTT	Forward	PCR
HuHVRr	CCG <u>ACGCGT</u> GCGGGCCGTCTGGTGGGTTATGGC	Reverse	PCR

^a Underlined nucleotides were required for cloning or mutagenesis purposes.

Chapter 5

General Conclusions

Hepatitis E is caused by infection with HEV, a non-enveloped, positive-sense, single-stranded RNA virus. HEV is transmitted via the fecal-oral route. Contaminated water and food supplies have been implicated in large epidemics of hepatitis E in Asia and Africa. Hepatitis E is a disease with a high attack rate in the adolescents and young adult population and has a higher incidence and mortality rate during pregnancy. Susceptibility of many animal species to HEV infection suggests the existence of animal reservoirs and the possibility of zoonotic spread of the virus. Both swine HEV and avian HEV are antigenically and genetically related to human HEV. HEV infections in pigs and chickens are being used as animal model systems to study the replication and pathogenesis of HEV. In this dissertation, both chicken and pig models as well as HEV reverse genetics systems were used to study the role of the hypervariable region (HVR) in the replication of HEV.

By comparing sequences of known HEV strains from different genotypes, we identified an HVR with a high degree of variability at both amino acid and nucleotide sequence levels. We found that the genotype 1 human HEV GFP replicon can tolerate partial deletions in HVR for its replication in Huh7 cells. Replication of HEV GFP replicon was confirmed by the expression of eGFP fluorescent signal in transfected Huh7 cells. To further confirm our results from *the in vitro* study, we constructed three avian HEV mutants with various deletions in the HVR and tested the mutants for their ability to infect chickens. We showed that avian HEV tolerated partial HVR deletions for its infectivity in chickens, whereas a larger deletion in HVR apparently attenuated the virus. In order to definitively verify our results from the chicken study, as well as the *in vitro* study, the infectivity of four HVR deletion-mutants of a genotype 3 swine HEV were subsequently analyzed in pigs. Similarly, mutants carrying partial deletions in the HVR were able to infect pigs, whereas the mutant with a larger or nearly complete HVR deletion was attenuated. The results suggest the presence of amino acid sequences in the HVR that are dispensable for HEV replication and infectivity.

Though HVR is a flexible domain with some residues that are not absolutely required for HEV infectivity, it may interact with viral and/or host factors to play more subtle roles in either viral replication or infectivity. To determine the role of HVR in viral replication, a genotype 1 human HEV replicon was constructed by replacing the ORF2 gene with the fire fly luciferase

reporter gene to quantify viral RNA synthesis *in vitro*. A series of deletions was made at the N-terminus, central region and the C-terminus of the HVR in the backbone of the HEV luciferase replicon. The ability of altered HVR to support viral RNA replication was tested by transfecting Huh7 cells. All the HVR-deletion mutants were replication competent *in vitro*. However, deletions in the HVR significantly reduced the level of viral RNA synthesis in Huh7 cells. To further examine the effects of HVR deletions on virus replication, a luciferase replicon for a non-mammalian strain of HEV, avian HEV, was constructed. The deletions were then engineered in the HVR of avian HEV to produce avian HEV HVR-deletion mutants. Following transfection in LMH chicken liver cells, we observed that viral RNA replication was considerably reduced by HVR deletions when compared to wild-type avian HEV. Relatively lower levels of virus replication were observed for HVR-deletion mutants when compared to wild-type HEV suggesting that HVR may play a possible role in RNA replication perhaps via specific interactions with viral and/or host-specific factors in the infected cells, which could modulate the efficiency of RNA replication. Deletions introduced in HVR may also interfere with the folding of neighboring functional elements and alter viral and/or host interactions necessary for efficient virus replication.

To analyze the impact of complete HVR deletion on the infectivity of avian HEV *in vivo*, an avian HEV mutant with a deletion of the entire HVR ($\Delta 557-603$) was generated using the avian HEV infectious cDNA clone as the backbone. After verifying the viability of HVR-deletion mutant in LMH cells by IFA, SPF chickens were intrahepatically inoculated with capped RNA transcripts generated from the HVR-deletion mutant. We found that the complete HVR-deletion mutant of avian HEV was non-infectious during the 5 weeks period of the study as evidenced by the absence of fecal shedding, viremia and seroconversion. It remains unclear why the avian HEV mutant with the entire HVR deletion is non-infectious *in vivo*, although it is likely that the drastic reduction of virus replication due to the entire HVR deletion renders the mutant non-infectious in chickens. It will be interesting to know if HVR contains critical residues that may play a role in antagonizing or evading immune mechanisms *in vivo*.

We also examined if HVR sequences are genotype-specific with respect to virus replication and infectivity. For this purpose, we swapped the HVR of genotype 1 human HEV with the HVRs of genotype 3 swine HEV and avian HEV. Similarly, the HVR of genotype 3 swine HEV was swapped with the HVR of genotype 1 human HEV. All the chimeras were tested

in Huh7 cells for viability, and the results revealed that all the three chimeras are replication-competent *in vitro*. To assess the impact of chimeras with swapped HVR on virus infectivity, we performed an infectivity assay using HepG2 cells. HepG2 cells were infected with lysates of transfected Huh7 cells collected at 9 days post-transfection, and IFA was performed to detect viral antigen. Except for the HepG2 cells infected with lysates from the chimera which contains the HVR of avian HEV, the other two chimeras were infective in HepG2 cells. Our results suggest that exchange of the HVR between mammalian HEV genotypes does not abolish virus replication-competency or infectivity, indicating that HVR is not genotype-specific for virus replication. In addition, the results also indicate that the HVR from an avian HEV is not compatible with the mammalian strains of HEV for infectivity.

In summary, the HVR has some amino acid sequences that are dispensable for HEV replication and infectivity. Critical amino acid residues, although not absolutely required for virus replication or infectivity, may play a role in enhancing the virus replication efficiency and/or infectivity of HEV. Future studies on the structure-functional relationship of HEV genes are warranted to clearly define additional roles of HVR and/or possible virulence determinants.