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REVIEW

Molecular biology and replication of hepatitis E virus

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Hepatitis E virus (HEV), a single-stranded, positive-sense RNA virus, is responsible for acute hepatitis E epidemics in many developing countries, and the virus is also endemic in some industrialized countries. Hepatitis E is a recognized zoonotic disease, and several animal species, including pigs, are potential reservoirs for HEV. The genome of HEV contains three open reading frames (ORFs). ORF1 encodes the nonstructural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a small multifunctional protein. The ORF2 and ORF3 proteins are translated from a single, bicistronic mRNA. The coding sequences for these two ORFs overlap each other, but neither overlaps with ORF1. Whereas the mechanisms underlying HEV replication are poorly understood, the construction of infectious viral clones, the identification of cell lines that support HEV replication, and the development of small animal models have allowed for more detailed study of the virus. As result of these advances, recently, our understanding of viral entry, genomic replication and viral egress has improved. Furthermore, the determination of the $T=1$ and $T=3$ structure of HEV virus-like particles has furthered our understanding of the replication of HEV. This article reviews the latest developments in the molecular biology of HEV with an emphasis on the genomic organization, the expression and function of genes, and the structure and replication of HEV.
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INTRODUCTION

Hepatitis E virus (HEV) is a small, non-enveloped virus with a single-stranded, positive-sense RNA genome that is approximately 7.2 kb in size.[1,2] HEV is transmitted mainly via a fecal–oral route and is the causative agent of hepatitis E, which is an important public health disease in many developing countries. Sporadic and cluster cases of acute hepatitis E have also been reported in many industrialized countries,[1,3,4] including Japan,[5] the United States,[6] and countries in Europe.[7] Unlike other forms of acute viral hepatitis, HEV infection is associated with a relatively high mortality rate (up to 30%) in infected pregnant women.[8–10] Four major genotypes of mammalian HEV are recognized; genotypes 1 and 2 are restricted to human infection, whereas genotypes 3 and 4 are zoonotic. Pigs are a known reservoir for HEV, and several other animal species, including deer, rabbits and mongooses, may potentially serve as HEV reservoir.[11–13] Although the currently available HEV cell culture systems are not robust,[14] they do support the full virus replication cycle. Therefore, these systems afford an opportunity to understand the mechanisms underlying cell entry, genomic replication, virion morphogenesis and viral egress in vitro.[9,15–17] Furthermore, the construction of infectious cDNA clones and replicons of HEV has paved the way for structural and functional studies of HEV genes,[18–22] and the development of pig- and chicken-based small animal models allows for the study of HEV replication and pathogenesis in homologous animal model systems.[23,24] Finally, the recent determination of the three-dimensional structure of HEV virus-like particles (VLPs)[25–29] has further advanced our understanding of the molecular biology and replication of this virus.

This article provides an overview of the recent advances in the understanding of the molecular biology and replication of HEV.

HOST RANGE AND HETEROGENEITY OF HEV

HEV is a member of the *Hepeviridae* family,[2,30] and two major species of HEV have been recognized. Mammalian HEV causes acute hepatitis in humans and has animal reservoirs in pigs and possibly other mammals,[12] whereas avian HEV is associated with hepatitis-splenomegaly syndrome in chickens[31] (Table 1). The species in the genus of *Hepevirus* includes the four major genotypes of mammalian HEV;[12,13] genotypes 1 and 2 have been identified in humans in developing countries, and genotypes 3 and 4 have been identified in humans and other animal species, including domestic and wild pigs, deer, mongooses and rabbits in both developing and industrialized countries. Recently, two putative new HEV genotypes were identified. One was identified in wild boars in Japan[32] and the other in rats in Germany[33,34] (Figure 1).

Avian HEV is currently classified as a separate, floating species in the *Hepeviridae* family.[2,30] Currently, at least three genotypes of avian HEV have been identified from chickens worldwide.[2,9,35,36] Because of the extensive sequence variation (approximately 50%) between avian and mammalian HEVs, it has been proposed to classify avian HEV as a new genus as opposed to its current classification as a floating species within the *Hepeviridae* family[9] (Figure 1).

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Table 1 Host range and heterogeneity of HEV

Proposed classification	Natural host	Susceptible experimental host	References
Orthohepevirus genus			
Genotype 1	Humans	Non-human primates, rats[a], lambs[a], rabbits[a]	23, 116–118
Genotype 2	Humans	Non-human primates	116, 119
Genotype 3	Humans, domestic pigs, wild boars, deer, mongoose, rabbits	Non-human primates, rabbits, domestic pigs	120–124
Genotype 4	Humans, domestic pigs, wild boars, cattle[a], sheep[a]	Non-human primates, domestic pigs, Balb/C mice[a], rabbits[a]	125–129
Novel unclassified genotype, Rat HEV	Rats	Unknown	34
Novel unclassified genotype, Boar HEV	Wild Boars in Japan	Unknown	32
Avihepevirus genus			
Genotype 1	Chickens (Australia)	Chickens	31
Genotype 2	Chickens (the United States, Canada)	Chickens, Turkeys	24, 75, 130
Genotype 3	Chickens (Europe, China)	Unknown	36, 131
Piscihepevirus genus			
Cutthroat trout virus	Brown, Apache and Gila trout	Unknown	37

Modified from Meng (2011).[13]
[a] Reported but not yet independently confirmed.

Cutthroat trout virus (CTV) from fish[37] also appears to be a member of the *Hepeviridae* family. Because of the low level of sequence identity to mammalian or avian hepeviruses (13%–27%), CTV likely represents another new genus in this family of viruses (Figure 1).

GENOMIC ORGANIZATION

The HEV genome is a single-stranded, positive-sense RNA molecule. Mammalian and fish HEV is approximately 7.2 kb in size, but avian HEV is approximately 6.7 kb in size.[38] The genome contains a short 5′ noncoding region (NCR), open reading frame (ORF) 1 that encodes the nonstructural proteins, ORF2 that encodes the capsid protein, ORF3 that encodes a small multifunctional protein, and a 3′ NCR[1] (Figure 2). The proteins encoded by ORF2 and ORF3 are produced from a bicistronic subgenomic (SG) mRNA. A junction region (JR) that is similar to the alphavirus JR sequence is present between ORF1 and the start site of the subgenomic coding region. Therefore, the coding region for ORF2 overlaps with ORF3, but neither overlaps with ORF1.[19,39] The 5′ end of the HEV genome contains a 7-methylguanosine cap structure, and the genome is organized as 5′ NCR-ORF1-JR-ORF3/ORF2-3′ NCR. Additionally, two *cis*-reactive elements (CRE) have been identified in the genome. The first CRE is essential for HEV replication and overlaps with both the 3′ end of ORF2 and the 3′ NCR.[40] The second CRE is located in the JR, and both the sequence and structure of the stem–loop (SL) in the JR are crucial for HEV replication.[22] Furthermore, the second CRE may be the promoter for the 2.0-kb SG mRNA.[19,41]

HEV PROTEINS AND THEIR FUNCTIONS

### Non-structural proteins (NSPs)

The HEV NSPs are encoded by ORF1. This coding region begins immediately after the 5′ NCR and extends 5082 bp (for the genotype 1 HEV Sar-55 strain). ORF1 encodes a 1693-amino acid (aa) polypeptide that is involved in viral replication and protein processing.[40] ORF1 contains several putative functional domains[1,42] (Figure 2), including a methyltransferase (Met) domain for capping the 5′ end of the viral genomic RNA,[43] a 'Y' domain of unknown function, a papain-like cysteine protease (PCP) domain, a proline-rich region that contains a hypervariable region (HVR), an 'X' domain of unknown function that is next to the PCP domain, a helicase (Hel) domain, and an RNA-dependent RNA polymerase (RdRp) domain that is responsible for viral replication.[44]

The Met domain is the first functional domain at the 5′ end of ORF1.[43] Both guaine-7-Met and guanyltransferase activities were detected in a baculovirus-expressed 110-kDa ORF1 polyprotein. Capping of the HEV genomic RNA was verified using a 5′ RNA ligase-mediated rapid amplification of cDNA ends method that can selectively amplify capped RNAs.[45] The cap is critical for viral infectivity, as only capped genomic RNA is infectious in non-human primate models.[40] A purified recombinant HEV Hel protein was shown to possess γ-phosphatase activity that may catalyze the first step in the formation of the cap,[46] suggesting that the Hel domain of the HEV protein is active and may be involved in the synthesis of the 5′ capped HEV RNA.

When expressed in *Escherichia coli* (*E. Coli*), the Hel domain of HEV possesses both nucleoside triphosphatase and 5′–3′ RNA duplex unwinding activities.[47] Mutations in the first walker A motif, including substitution of the K residue of the nucleotide-binding motif with an A residue, can lead to a 70% loss in ATPase activity. Similarly, mutating the DE residues in the Mg^{2+}-binding motif present in the second walker B motif to aa residues caused a 50% reduction in ATPase activity. Mutations in both motifs abolished the Hel activity of the protein.

A putative PCP domain has been predicted in HEV ORF1;[42] however, definitive evidence for the translational and post-translational processing of the ORF1 polyprotein is still lacking. HEV ORF1 has been expressed as a 185-kDa polyprotein in *E. coli*, insect cells and mammalian cells.[48–50] When ORF1 was expressed in mammalian cells using a vaccinia virus-based expression system, two potential post-translational processing products of 107 and 78 kDa were observed only after an extended incubation time.[49] However, disruption of the predicted protease catalytic site in the ORF1 PCP domain did not affect the processing of the protein products. This suggests that the observed cleavage products are not generated by a viral protease and that they are likely produced by the expression system that was used in the study. Additionally, when HEV ORF1 was expressed in a baculovirus expression system, multiple smaller proteins were seen, and the presence of the putative processing products was abolished after the addition of the cell-permeable cysteine protease inhibitor E-64d.[51] Therefore, conclusive evidence detailing the processing of the ORF1 protein in the baculovirus system is still lacking.

More recently, the deconjugation activity of a purified recombinant protein that contained the Met and the papain-like cysteine protease
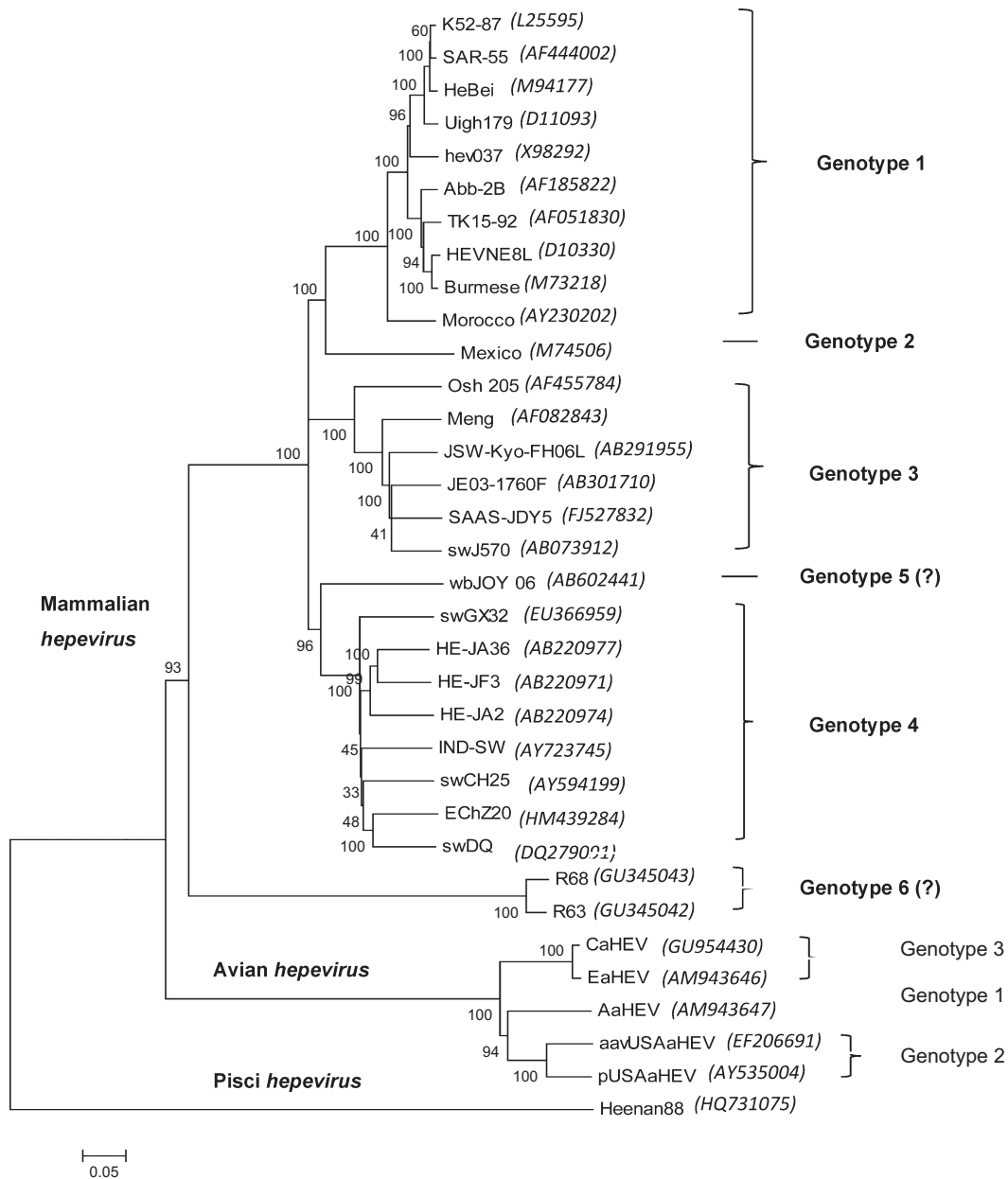
Figure 1 A phylogenetic tree based on the complete genomic sequences of representative HEV strains from each genotype. The tree was constructed using the neighbor-joining method with 1000 bootstrap analyses using MEGA5. The scale bar represents 0.05 nucleotide substitutions per position. The bootstrap values are labeled at the major nodes. The GenBank accession numbers of the HEV genomes are included in parenthesis after each strain.

domains (MetT-PCP) from ORF1 was analyzed using the following fluorogenic substrates: ubiquitin-7-amino-4-methylcoumarin (Ub-AMC), interferon-stimulated gene 15 (ISG15)-AMC, neural precursor cells expressed developmentally downregulated 8 (Nedd8)-AMC and small ubiquitin-like modifier (SUMO)-AMC. The MetT-PCP recombinant protein hydrolyzed all four substrates, and the hydrolysis of ISG15-AMC was significantly greater compared with the other substrates, suggesting that the HEV MetT-PCP recombinant protein possesses deubiquitinating activity.[52] However, processing of the Hel and the RdRp domains that contained the conserved LXGG site (position 1205) was not observed. Thus, it remains unclear whether the ORF1 polyprotein functions as a single protein with multiple functional domains or if the cleaved smaller proteins function independently.

The viral RdRp contains eight conserved motifs (motif I–VIII) that are similar to the RdRps from other positive-sense RNA viruses.[53] The GDD sequence is essential for HEV replicase activity, as demonstrated by mutation of an enhanced green fluorescent protein (EGFP)-containing HEV replicon.[54] The HEV RdRp can bind to the 3′ NCR of the HEV genome, and when expressed in *E. coli*, recombinant HEV RdRp can synthesize RNA *in vitro* using the 3′ polyadenylated HEV RNA as a template in a primer-independent manner.[44] Furthermore, the RdRp is localized to the endoplasmic reticulum (ER) of the cells that express RdRp recombinant protein,[55] suggesting that the ER may be involved in HEV replication.

An HVR was identified in ORF1 of HEV. The HVR overlaps the proline-rich sequence that is located between the N terminus of the

Genomic RNA
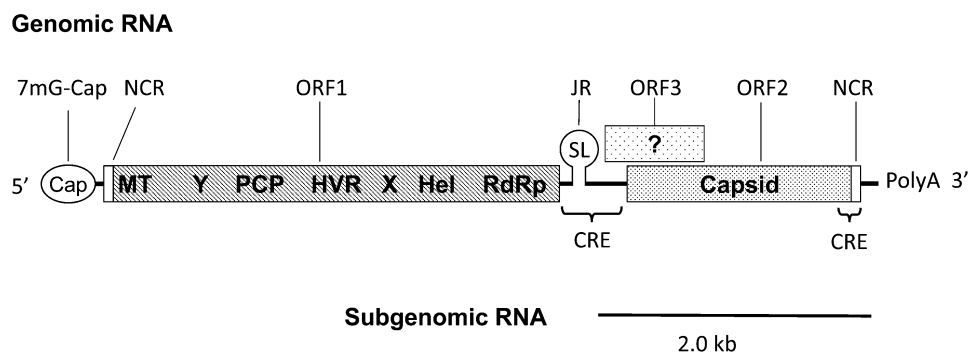
Figure 2 A schematic diagram of the genomic and subgenomic organization of the HEV genome. The three ORFs are labeled and shown as boxes with the putative ORF1 domains indicated inside the box. Modified from Cao *et al*. (2010).[22] CRE, *cis*-reactive element; Hel, helicase; HEV, hepatitis E virus; HVR, hypervariable region; JR, junction region; MT, methytransferase; NCR, noncoding region; ORF, open reading frame; PCP, a papain-like cysteine protease; RdRp, RNA-dependent RNA polymerase; SL, stem–loop structure; X, macro domain; Y, Y domain.

X domain and the C-terminal portion of the putative PCP domain.[42] The HVR varies both in length and sequence among different HEV strains.[56] It has been demonstrated that HEV can tolerate small deletions in the HVR and that the aa residues in this region are dispensable for viral infectivity.[56] However, the replication of HVR deletion mutants was markedly reduced in Huh7 cells, and a similar reduction in HEV replication was observed when HVR deletion mutants from avian HEV were tested in LMH chicken liver cells.[57] The HVR sequences are functionally interchangeable between different HEV genotypes with respect to viral replication and infectivity *in vitro*; however, genotype-specific HVR differences in replication efficiency have also been observed.[57] This suggests that while small deletions in the HVR do not have a significant impact on viral infectivity, the HVR may influence the efficiency of HEV replication by interacting with viral and/or host factors. Interestingly, a rare, recombinant HEV that contained a 174-base insertion from a human ribosomal protein gene was isolated when the Kernow-C1 strain of HEV, which was originally isolated from a human immunodeficiency virus-infected individual, was adapted to grow in human hepatocellular carcinoma cells.[15] The insertion in the HVR region was stable during serial passages in cell culture, and using an infectious cDNA clone, this insertion was found to be a key factor in the adaptation of the virus to growth in cell culture. Both the aa composition and the size of the insertion are important in viral replication.[58]

Recently, it has been demonstrated that chimeric viruses that contain the ORF2 capsid gene either alone or in combination with the adjacent 5′ JR and 3′ NCR from a genotype 4 human HEV in the backbone of a genotype 3 swine HEV are replication competent in Huh7 cells, infectious in HepG2/C3A cells, and infectious in pigs. However, chimeric viruses that contain the JR, ORF2 and 3′ NCR from zoonotic genotypes 3 or 4 HEV that infect humans and pigs in the backbone of genotype 1 human HEV failed to infect pigs. This suggests that the 5′ NCR and ORF1 may be involved in cross-species HEV infection or may be related to host tropism.[59]

The capsid protein

The HEV capsid protein is encoded by ORF2 and is 660 aa in length with a molecular weight of approximately 72 kDa. The capsid protein has a typical arginine-rich signal peptide sequence and three potential N-linked glycosylation sites.[1,9] When expressed *in vitro*, capsid proteins of various sizes have been observed.[60,61] In cells that are infected with recombinant Semliki Forest virus that contains ORF2, two larger ORF2-specific proteins were identified that were likely due to

N-linked glycosylation.[62] However, when the HEV ORF2 protein was expressed in a baculovirus system, four capsid protein products of 72, 63, 56 and 53 kDa were observed. Sequence analyses revealed that the 53-, 56- and 63-kDa proteins were cleaved products from the full-length 72-kDa protein.[63]

The ORF2 capsid protein is involved in the assembly of the HEV particle and its interaction with host cells. The capsid protein contains a potential ER localization signal at its N terminus, and while the protein does enters the ER, a fraction of the protein retrotranslocates to the cytoplasm.[64] The majority of the capsid protein expressed in mammalian cells is glycosylated, and the glycosylation is important for the formation of infectious virus particles.[20] The capsid also binds to HEV genomic RNA[65] and may play a role in viral assembly. The capsid interacts with host cells and binds to heat shock protein 90 (HSP90),[66] glucose-regulated protein 78 (Grp78)[67] and heparin sulfate proteoglycans (HSPGs).[68] It has been demonstrated that HSPGs may serve as an attachment receptor to facilitate HEV entry into the host cells, whereas Grp78 or HSP90 may be involved in intracellular transport. Recently, it has been shown that three aa mutations (F51L, T59A and S390L) in the HEV ORF2 capsid protein[21] result in viral attenuation. These three mutations significantly reduced viremia, delayed the onset time of viremia, shortened the duration of fecal virus shedding and viremia, and reduced the viral loads in the liver, bile and intestinal contents.[69]

The HEV ORF2 capsid protein is immunogenic, and neutralizing antibodies have been shown to target it. Both linear and conformational epitopes for neutralizing antibodies have been mapped to aa residues 578–607,[70] 452–617,[71] and 458–607[72] in the C-terminal portion of ORF2. Recent studies demonstrated that only antibodies recognizing conformational epitopes are neutralizing, and the aa residues Leu477 and Leu613 in the capsid protein are important in forming a neutralization-sensitive epitope.[73] Recently, the epitope of the 8C11 neutralizing antibody was identified using the complex structure of the HEV E2s domain and the 8C11 antibody. The neutralizing epitope was found to be conformational and located at aa residues 496–499, 510–514 and 573–578.[74] Western blot analysis showed that the recombinant avian HEV capsid protein reacted with both antisera against genotype 1 human HEV and convalescent antisera against genotype 3 swine and human HEVs. Similarly, convalescent sera from chickens that were experimentally infected with avian HEV reacted with the recombinant capsid proteins from the genotype 3 swine HEV and genotype 1 human HEV.[75] The convalescent serum from animals that were infected with any of the four mammalian genotypes of HEV all neutralized genotype 1 HEV.[76] Despite the broad antigenic

cross-reactivity between the known HEV genotypes, antigenic variations have been observed among HEV strains using genotype- and strain-specific monoclonal antibodies.[77] Therefore, both pan-genotypic and strain-specific epitopes exist. It has been reported that aa residue 606 in the capsid is important in maintaining the antigenicity of the HEV ORF2 protein.[77] The E2 domain of the capsid protein, including aa residues 455–602, forms a homodimer. A strong hydrophobic cluster at the dimer interface is maintained by the side chains of residues Val503, Trp548, Thr552, Ala555, Tyr557, Tyr561, Val598 and Val600 in both subunits of the dimer. The C-terminal residues Ala597, Val598, Ala599, Leu601 and Ala602 are also involved in the formation of the dimmer.[26]

The cytoskeleton-associated multifunctional phosphoprotein

HEV ORF3 encodes a small, 114-aa, cytoskeleton-associated phosphoprotein that is translated from the bicistronic SG RNA. At its 3′ end, ORF3 overlaps ORF2 by approximately 300 nt, but it does not overlap ORF1.[19,39] The protein encoded by ORF3 contains two N-terminal hydrophobic domains (D1 and D2) and two C-terminal proline-rich regions (P1 and P2).[17] The phosphorylated ORF3 protein interacts with the non-glycosylated capsid protein via a 25-aa region in the ORF3 protein. This ORF2–ORF3 interaction depends on the phosphorylation of the ORF3 protein at the Ser71 residue and may play a regulatory role in the assembly of HEV virions.[78] The C-terminal region of the ORF3 protein is multifunctional and appears to be involved in virion morphogenesis and pathogenesis.[78–80]

When expressed in mammalian cells, the ORF3 protein is approximately 13 kDa in size, it is phosphorylated at the Ser71 residue, and it is associated with the cytoskeletal and membrane fractions.[81] Similarly, ORF3 proteins were also detected in cells that were transfected with the HEV genomic RNA.[18,41,82] The expression of the ORF3 protein displays both filamentous and punctate distribution patterns. The filamentous distribution reflects the interaction between the ORF3 protein and microtubules,[83] whereas the punctate distribution is associated with both early and recycling endosomes.[84] Using an in vitro replicon system, it was shown that the ORF3 protein is not required for HEV replication in Huh7 cells and other cell lines,[18] but the ORF3 protein is essential for both infection of rhesus macaques[41] and the release of virions from infected cells.[16,85] Monoclonal antibodies against the ORF3 protein can capture HEV particles from cell culture supernatants and serum from HEV-infected patients but cannot capture fecal virus. This suggests that the ORF3 protein is present on the surface of nascent HEV virions.[86]

It has been shown that the ORF3 protein colocalizes with the cytoskeleton via interactions through the ORF3 D1 hydrophobic domain,[81] and it binds to mitogen-activated protein kinase (MAPK) phosphatase[87] and microtubules.[83] The D2 hydrophobic domain interacts with hemopexin and affects cellular iron homeostasis.[88] The P2 region contains a proline-rich PxxP motif and binds to sarcoma(src)-homology 3 (SH3) domain-containing proteins,[89] which have important functions in signal transduction pathways that promote cell survival.[90] It has been reported that the ORF3 protein activates extracellular signal-regulated kinases (Erks)[89] via the binding and inactivation of an Erk-specific MAPK phosphatase, Pyst1. The ORF3 protein binds Pyst1 through its D1 domain and blocks conformational changes in Pyst1 that are required for Erk-mediated activation.[87] It has also been reported that the ORF3 protein upregulates the expression of enzymes in the glycolytic pathway via the stabilization of hypoxia-inducible factor 1 (HIF-1),[91] and it interacts with the fibrinogen (FBG) Bβ chain.[92]

The ORF3 protein inhibits the mitochondrial apoptosis pathway[93] and delays the transport of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) into the cell nucleus.[84] The ORF3 protein also delays the trafficking and degradation of the activated hepatocyte growth factor receptor (c-Met). Because of its effects on growth factor receptor trafficking, it is believed that the ORF3 protein promotes cell survival.[94] The ORF3 protein has also been shown to interact with α1-microglobulin and bikunin.[79,80] In cells that express the ORF3 protein, there is an increase in the secretion of α1-microglobulin,[95] which is mediated by tumor susceptibility gene 101 (Tsg101). Recent studies have revealed that the ORF3 protein binds to Tsg101 via its PSAP motif,[95,96] and that the ORF3 protein interacts with Tsg101 and α1-microglobulin to facilitate the assembly of the endosomal sorting complex required for transport (ESCRT) complexes.[95] It has also been reported that Tsg101 and the vacuolar protein-sorting (Vps) proteins Vps4A and Vps4B are involved in release of HEV particles. This suggests that the multivesicular body (MVB) pathway is involved in viral release.[97] It appears that the ORF3 PSAP motif is a late domain involved in the release of membrane-associated HEV particles from infected cells. The interactions between the ORF3 protein and host proteins may create a favorable environment for HEV replication and pathogenesis. In Huh7 liver cells, it has been demonstrated that ORF3 increases the phosphorylation of hepatocyte nuclear factor 4 (HNF4). Phosphorylation impairs the nuclear translocation of HNF4 and downregulates HNF4-dependent gene expression.[98] Taken together, these results suggest that the ORF3 protein is a multifunctional protein that appears to play an important role in HEV replication and pathogenicity.

CRES IN HEV GENOME

Single-stranded, positive-sense RNA viral genomes generally form secondary and tertiary structures, known as CREs, that maintain the stability of the viral RNA genome and participate in molecular interactions. The CREs are involved in RNA–RNA interactions and the binding of viral and cellular proteins during viral replication. CREs are generally located in the conserved 5′ and 3′ NCRs of the viral genomes. However, the 5′ NCR of the HEV genome is only 26 nt long, has a cap structure and plays a role in the initiation of HEV replication.[40,43,45] No CRE has been identified in the 5′ NCR of the HEV genome.

The first CRE that was identified in the HEV genome is important for HEV replication, is located in the 3′ NCR, and overlaps with the 3′ end of the coding region for ORF2.[40] The 3′ NCR and an adjacent region form two SL structures (SL1 and SL2) (Figure 3A) that both interact with the viral RdRp.[44] This interaction is sequence specific, because 3′ NCR deletion mutants that lack SL1, SL2 or the polyA failed to form a complex with the viral RdRp. This finding suggests that the 3′ end of the viral genome, including the SLs and the polyA stretch, is recognized by the viral RdRp. It has also been reported that despite the sequence variation in the 3′ termini of the different HEV genotypes, the replication of chimeric HEV genomes and replicons generated from different HEV genotypes is similar to the replication of the parental virus or replicon. This result suggests that viral replication is not significantly affected by swapping the 3′ NCR between different HEV genotypes.[99] However, it appears that both the structure and the sequence of the SLs in the 3′ NCR are critical for viral replication. A single nucleotide change that disrupts one base pair within SL2 at position 7106 in genotype 1 HEV significantly decreased viral replication.[40,99] Moreover, both a short hairpin RNA (shRNA)[100] and a ribozyme[101] targeting the 3′ NCR of HEV effectively inhibited viral
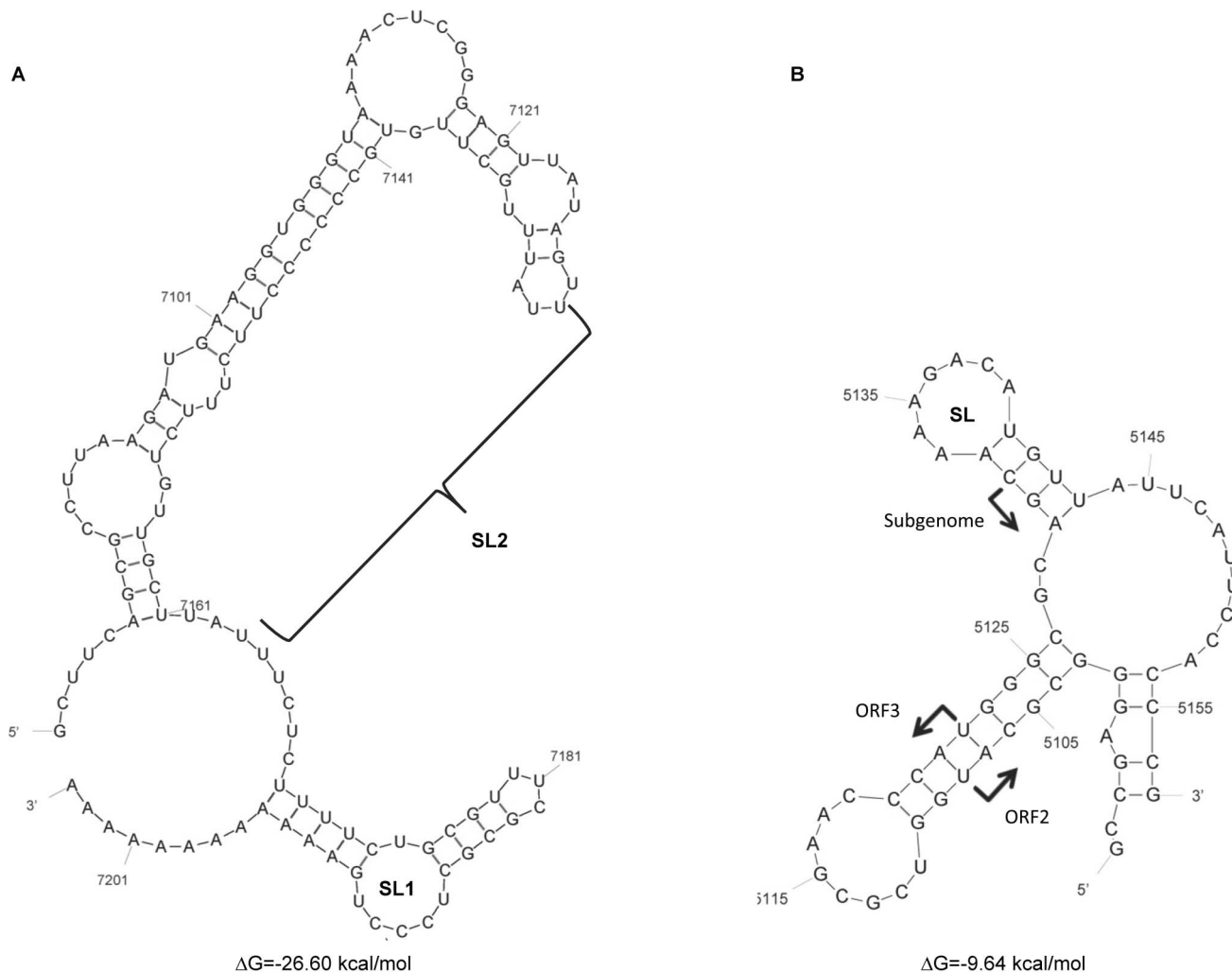
Figure 3 The secondary structure of the CREs of a genotype 1 HEV (strain Sar-55) as predicted using mfold. (**A**) The secondary structures at the 3' end of HEV genomic region (nt 7082–7192 A^n) with the SL1 and SL2 labeled.[40,44] (**B**) Negative-polarity complement of the JR in the HEV genome (nt 5096–5157).[22] The regulatory SL is labeled, and the arrows indicate the start sites of the subgenomic mRNA, ORF2 and ORF3.

replication, further indicating that the 3′ NCR is important in HEV replication.

The second HEV CRE is located in the JR of the HEV genome. This CRE may serve as a promoter for the synthesis of the 2.0-kb SG mRNA.[22,39,41] It has been shown that when 6-nt or 4-nt mutations were introduced into the JR, neither ORF2 nor ORF3 protein expression was detectable *in vitro*,[41] suggesting the presence of a CRE in the JR. Subsequently, a highly conserved SL structure was identified within the HEV JR (Figure 3B). The JR shares nucleotide sequence identity with a JR in a Rubella virus and with the conserved alphavirus SG promoter sequence.[39] The role of the SL structure within the JR in HEV replication was demonstrated using Huh7 cells that were transfected with either wild-type or SL mutant replicons that contained a *Renilla* luciferase reporter.[22] Previous research has demonstrated that mutation of the AGA motif in the loop structure significantly reduced the levels of HEV replication. Additionally, mutations in the stem of the structure and in the subgenomic initiation site also significantly inhibited HEV replication. These results indicate that both the sequence and the SL structure in the JR are important for HEV replication. Delineating the exact role of the HEV RNA in viral replication will facilitate an increased understanding of the life cycle of the virus.

VIRION STRUCTURE AND FUNCTION

When expressed in insect cells, the N-terminal, truncated capsid protein can self-assemble into VLPs of two different sizes, $T=1$ and $T=3$.[29,102,103] The $T=1$ VLPs (270 Å in diameter) are smaller than native virions (320–340 Å) but have similar antigenicity and a similar surface structure.[104,105] The crystal structures of $T=1$ VLPs of HEV genotype 1,[29] genotype 3[28] and genotype 4[25] have been determined, and a cryo-EM structure of $T=3$ VLPs has also been resolved.[29]

Despite significant sequence differences between the capsid proteins of different HEV genotypes, the $T=1$ VLP structures that are derived from HEV genotypes 1, 3 and 4 are very similar.[25,28,29] The $T=1$ VLP structure is an icosahedral particle that consists of 60 identical capsid protein subunits.[29] Three distinct domains have been identified in the $T=1$ VLP structure, the S (Shell), M (middle) and P (protruding) domains.[25,28,29] The S domain is a jelly roll-like β-barrel fold and is conserved among many small RNA viruses.[25] The M domain comprises a twisted β-barrel with six antiparallel β-strands and four short α-helices. This domain binds to and interacts with the S domain.[25,28] The P domain forms a dimeric spike that protrudes from the two-fold axis of symmetry in the $T=1$ VLPs.[25] The M and P domains are linked by a flexible, proline-rich hinge that facilitates

the topological changes in the protruding spike in the $T=1$ VLPs.[25,29] Dimeric, trimeric and pentameric interactions around the two-, three- and five-fold icosahedral axes are required for packaging of $T=1$ VLPs. The P domain forms a dimeric spike[26] that functions as the viral receptor, is the site of neutralizing antibody binding,[25,28,29] and is important for antigenicity.[106]

The Asn137 N-linked glycosylation site is partially hidden near the inner surface of the S domain, and the Asn310 N-linked glycosylation site is completely hidden by the interface of the capsid trimer. The Asn562 site is likely glycosylated in the ER.[28] It is likely that both the M and P domains are involved in virus entry.[28] A structure of $T=1$ VLPs in complex with the Fab224 neutralizing antibody has recently been resolved,[106] and this structure demonstrated that Fab224 recognizes a lateral surface of the P domain. Furthermore, it has been shown that insertion of an antibody epitope at the C terminus of the capsid protein does not interfere with the assembly of $T=1$ VLPs. This result indicates that $T=1$ VLPs may be good candidates for vaccine development[104] and may be useful as a potential oral vaccine vector.[106,107]

The HEV virion has also been predicted to have a $T=3$ icosahedral symmetry composed of 180 copies of the capsid protein.[25,105] Recently, virion-sized $T=3$ VLPs were purified from insect cells that expressed HEV ORF2 with 13-aa residues truncation at the N terminus.[29] The cryo-EM-derived structure of the $T=3$ VLPs indicated that 180 copies of the capsid protein are assembled, thus confirming the previous prediction.[29] The capsid proteins of the $T=3$ VLP are grouped into three unique monomers, the A, B and C monomers. The A and B monomers form dimeric spikes around each of the five-fold icosahedral axes of symmetry, whereas the C monomers form a dimeric spike at each of the two-fold icosahedral axes of symmetry.[29] The N-terminal aa residues 14–111 are thought to prevent the ORF2 proteins from assembling into $T=1$ VLPs.[29] While $T=1$ structures have been described, it is believed that the native HEV capsid is a $T=3$ icosahedron.
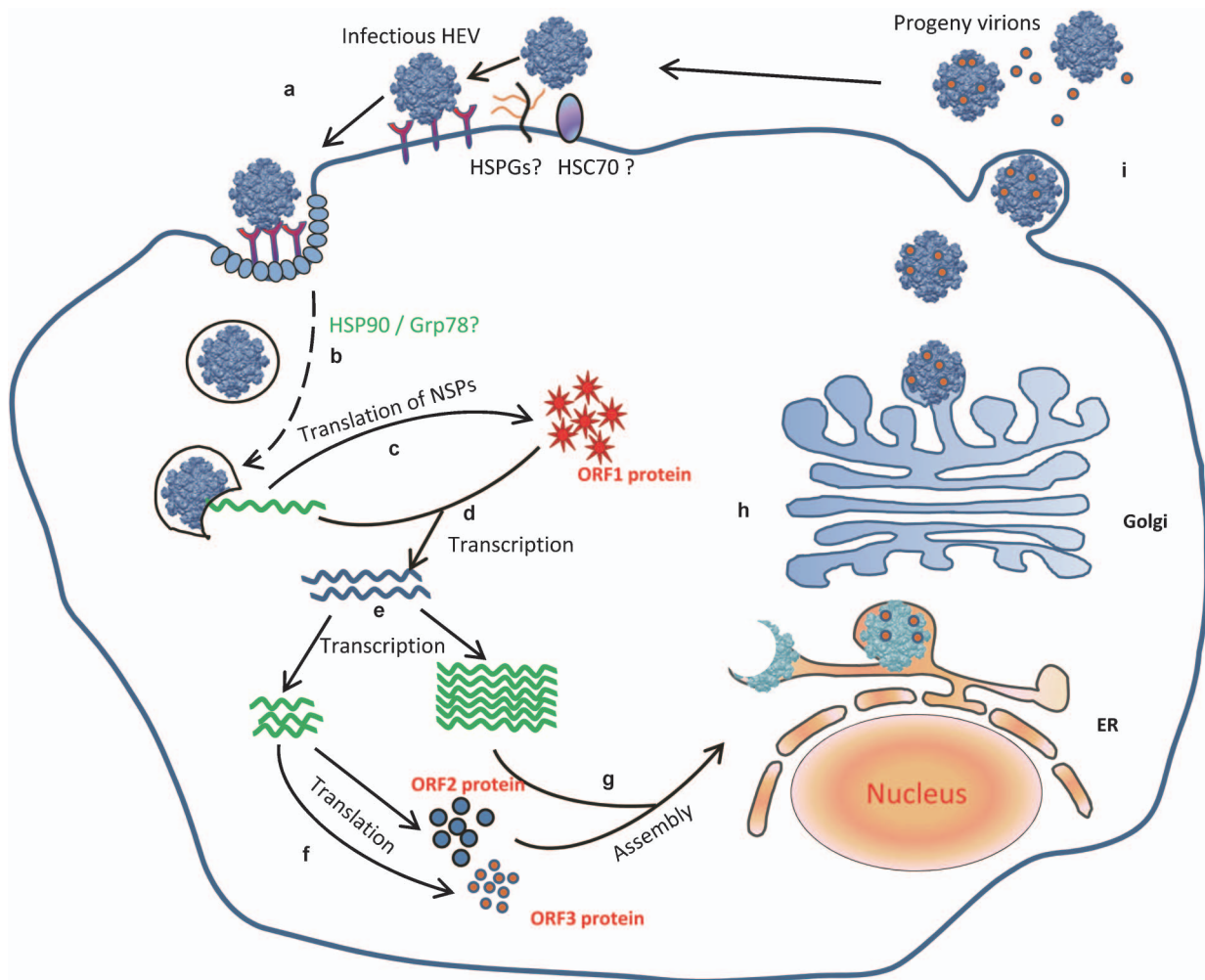
Figure 4 Proposed life cycle of HEV.[9,17,108,109] Step a: HEV attaches to the cell surface via HSPGs, HSC70 or other putative attachment receptor(s) and then enters the cell via a unknown specific cellular receptor.[68,110] Step b: The HEV virion penetrates the membrane and enters the cells. HSP90 and Grp78 may be involved in this transport. The virion then uncoats and releases the positive-sense genomic RNA into the cytoplasm of the cell. Step c: The positive-sense genomic viral RNA serves as the template to translate the ORF1 nonstructural polyprotein in the cytoplasm. Step d: The viral RdRp synthesizes an intermediate, replicative negative-sense RNA from the positive-sense genomic RNA that (step e) serves as the template for the production of positive-sense, progeny viral genomes. Step f: The ORF2 and ORF3 proteins are translated from the subgenomic, positive-stranded RNA, and (step g) the ORF2 capsid protein packages the genomic viral RNA and assembles new virions. Step h: The nascent virions are transported to the cell membrane. The ORF3 protein facilitates the trafficking of the virion, and (step i) the nascent virions are released from the infected cells. Modified from various studies.[17,108,109]

LIFE CYCLE OF HEV

Due to the lack of a robust cell-culture system for HEV, the life cycle of HEV is largely unknown.[17,108,109] The HEV capsid protein is believed to bind to a cellular receptor to initiate viral entry and replication. ORF2 peptide-binding experiments suggested that the C-terminal region of ORF2 may mediate virus entry by binding to heat shock cognate protein 70 (HSC70) on the cell surface.[110] Additionally, HSPGs have been identified as attachment receptors that are located on the cell surface[68] (Figure 4). As a 23-nm particle, a truncated dimer of the HEV capsid protein (HEV239) potentially binds to the HEV receptor(s) on cells.[111] A potential sugar binding sequence is located in the M domain. This sequence forms a hidden pocket at the interface between the two capsid molecules around the three-fold axis of symmetry. The putative binding motif on the capsid is conserved between all four major mammalian HEV genotypes, suggesting a functional role of this domain in binding to the cellular receptor.[25] It has been reported that the truncated HEV 239 capsid protein also binds to Grp78, which is a cellular chaperone. This binding may play an important role in the HEV life cycle.[67] However, a specific cellular receptor for HEV still remains elusive. After virus entry into permissive cells, the HEV genomic RNA is uncoated by unknown mechanisms. An HSP90-specific inhibitor (geldanamycin) blocks the intracellular transport of the HEV239 capsid protein but does not affect the binding and entry of the truncated capsid protein. This suggests that HSP90 may play a role in the intracellular transport of HEV.[66]

After uncoating of the viral genome and release of the viral genome into cells, the 7-methylguanosine cap structure in the 5′ NCR of the HEV genome recruits the 40S ribosomal subunit to initiate cap-dependent translation of viral proteins. The NSPs are translated from the genomic RNA. A negative-sense, replicative intermediate RNA is likely produced by the viral RdRp. This intermediate serves as the template for the production of positive-sense, progeny viral genomes. The activity of the HEV RdRp has been demonstrated in the HEV replicon systems,[44,99] and the negative-sense intermediate viral RNA has been detected in tissues from HEV-infected animals.[112–114] The RdRp may bind to the CRE in the 3′ NCR of the HEV genome,[44] which could play an important role in HEV replication.[40,99] The CRE in the JR of the HEV genome is important in the synthesis of the SG RNA.[22,41] This SG RNA is translated to produce the structural capsid protein and the small ORF3 protein.

Both the assembly and release of HEV are poorly characterized. It is believed that the ORF2 protein packages the viral genome and has a role in the assembly of progeny virions. A 76-nt region at the 5′ end of the HEV genome has been identified as a binding domain for the ORF2 protein, and this region may be involved in the packaging of the HEV genome.[65] The ORF3 protein is believed to be involved in viral egress.[16,115] The PSAP motif in the ORF3 protein is a late domain that is important for the release of the membrane-associated HEV particles from infected cells.[97]

In summary, HEV is an important but grossly under studied pathogen. The HEV life cycle is poorly understood, and often, there are more questions than answers for many of the various steps in its replication. However, over the past decade, significant advances have been made in many aspects of HEV growth and replication, including the determination of the three-dimensional virion structure, the establishment of infectious cDNA clones, the identification of animal strains of HEV, and the development of small animal models. However, as most of the available molecular biology data are derived from cells over expressing individual viral proteins, the biological significance of these data in the context of virus infection is mostly unknown. Therefore, future studies that aim to delineate the structure and function of the HEV genes, to understand the molecular mechanisms of HEV replication, and to examine virus–host interactions are warranted. The recent development of a more efficient cell culture system for HEV replication will aid in the future study of HEV molecular biology.

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