The Hepatitis E Virus Capsid C-Terminal Region Is Essential for the Viral Life Cycle: Implication for Viral Genome Encapsidation and Particle Stabilization

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Although the C-terminal 52 amino acids (C52aa) of hepatitis E virus (HEV) capsid are not essential for morphology, the C52aa-encoding region is required for replication. Transfection of a C52aa knockdown mutant showed transient growth, and the earliest population included a majority of noninfectious (possibly empty) particles and a minority of infectious particles with C-terminal capsid degradation. Finally, the complete revertant was generated reproducibly. C52aa is essential for the viral life cycle, promoting accurate encapsidation and stabilizing encapsidated particles.

Hepatitis E virus (HEV) is responsible for acute and enterically transmitted hepatitis in the developing world (1). Before the establishment of high-efficiency HEV cell culture systems (2), *in vitro* generation of HEV virus-like particles (HEV-VLPs) in insect cells and *in vivo* propagation in nonhuman primates were the most useful models for the study of HEV. Genetic deletions or cellular processing resulting in the loss of the N-terminal 111 or 13 amino acids (aa) or of the C-terminal 52 aa (C52aa) yielded capsid protein capable of directing the formation of the HEV small (S) or large (L) VLPs (3–5). Particle formation was required for C52aa abbreviation, limiting the structural analysis of the resulting particles (3, 4, 6–10). However, the contribution of the C52aa-encoding sequence was confirmed by both *in vivo* (attenuated infectivity of the point mutant virus in nonhuman primates) and *in vitro* (reduced RNA synthesis by RNA-dependent RNA polymerase [RdRp]) assays (11–14). Furthermore, the highly conserved nature of the C52aa sequence implies that the C52aa domain itself is functionally important. In this study, we characterized the role of the C52aa domain in the HEV life cycle by using infectious clones.

We constructed infectious clones by using the infectious virus G3-HEV83-2-27, employing a procedure described previously (20). Using a synthetic cDNA as the template, we amplified 12 fragments covering the entire G3-HEV83-2-27 genome by PCR with the primers listed in Table 1. These fragments were ligated stepwise and were inserted into the EcoRI-HindIII site of pUC19, yielding a wild-type clone that we designated WT. Site-directed mutagenesis of WT was used to generate clones that were mutated to encode capsid protein lacking the C52aa domain, either by introduction of an amber stop codon, UAA (a knockdown mutant designated Amut), or via deletion of the corresponding segment of the open reading frame 2 (ORF2) sequence (a knockout mutant designated Dmut). We performed experiments on three separate scales (normal, large, and huge, as described below) in order to estimate virus progeny productivity, to clarify the growth kinetics, and to analyze the process of encapsidation in the absence of revertants.

Normal scale. To estimate the virus progeny productivity of HEV without C52aa, transfection with Amut and Dmut was performed in comparison to transfection with WT. A 50- μ g quantity of RNA from each infectious clone was electroporated into 1×10^7 cells of PLC/PRF/5. Analysis by enzyme-linked immunosor-

bent assay (ELISA) (using an anti-G3-HEV-VLP rabbit polyclonal antibody [5]) suggested that transient growth was observed with Amut, in contrast to continuous growth with WT (Fig. 1A) and no growth with Dmut (data not shown). The productivity (expressed as the genome copy number) of Amut, measured by real-time reverse transcription-PCR (RT-PCR) of RNA with a set of specific primers (Table 1), was estimated as approximately 40-fold lower than that of WT (Fig. 1B and 1C; note the differences in scale). However, subsequent analysis demonstrated that the Amut-derived HEV actually harbored synonymous and nonsynonymous reversion mutations, suggesting that the actual productivity (of intact Amut) was much lower than that suggested by real-time RT-PCR. To assess the progeny, sucrose density gradient analysis (SDGA) (with a gradient from 10 to 60% [wt/vol] sucrose) was performed. Subsequently, the collected fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot analysis (WB) was performed with the polyclonal antibody noted above (5). Chemiluminescence was recorded using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). In the series of fractions obtained from progeny derived from infection with WT, the presence of antigen was confirmed only in fraction 8 (F8) in Fig. 1C by WB (data not shown). The 72-kDa size of the prominent band was in agreement with the size of the capsid protein predicted for the WT clone. Quantification of the HEV RNA genome copy number showed a trailing peak for the progeny derived from infection with Amut (Fig. 1B, F8 and F9) and a single peak for the progeny derived from infection with WT (Fig. 1C, F8). These peaks corresponded to similar specific densities. Sequence analysis showed that while the progeny from infection with WT carried the original sequence, the progeny from infection with Amut did not contain the expected UAA (amber codon) at this position. Instead, the trailing peak of this Amut-derived sample corresponded to two

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TABLE 1 Primers used for the construction of an HEV infectious cDNA clone, the C52aa deletion mutant, and the amber mutant and for the quantification and sequencing of HEV RNA by real-time RT-PCR

Name	Polarity ^a	Sequence (5'–3')	Position in genome (nt) ^b	Amplicon (amplified region in genome [nt]) ^b
ET7G2-F	+	<u>GAATTCAATACGACTCACTATAGGCAGACCACGTATGTGGTCGAT</u> ^c	2–23	Fragment 1-1 (2–155)
155R-EV	–	AGTCTGCACGCGAGATAAAAAACGGCCGGAC	126–155	
126F-EV	+	GTCCGGCCGTTTTATCTCGCGTGCAGACT	126–155	Fragment 1-2 (126–1370)
1370R-EV	–	CACCTGGGATCCAGATGGAAGCCCGCAG	1342–1370	
1363F-EV	+	TCTGCGGGCTTCCATCTGGATCCCAGGGTG	1341–1370	Fragment 2-1 (1341–1794)
1816R-EV	–	ACTGCTCAGGGCCGTTCGCCCTCAAGATGAG	1765–1794	
1787F-EV	+	CTCATCTTGAGGCGAAGCGCCCTGAGCAGT	1765–1794	Fragment 2-2 (1765–2934)
2956R-EV	–	CGGCACAGGCACGGCCAACCTCTGTGGCAG	2905–2934	
2857F-EV	+	CCGATGCAGCCGGCACTCACAATAACGGAG	2835–2864	Fragment 3-1 (2835–3194)
3216R-EV	–	AGCCCGCTGCATATGTAATAGCAGCAAGTG	3165–3194	
3187F-EV	+	CACCTTGCTGCTATTACATATGCAGCGGGCT	3165–3194	Fragment 3-2 (3165–3925)
3947R-EV	–	TCCGTAAGCTCAAAAACCAACACACTATCG	3896–3925	
3918F-EV	+	CGATAGTGTGTGGTTTTTGGAGCTTACCGA	3896–3925	Fragment 3-3 (3896–4598)
4620R-EV	–	CTTCCAAAACCCCTTAAGGGATTCTCTTAGG	4569–4598	
4591F-EV	+	CCTAAGGAATCCCTTAAGGGGTTTTGGAAG	4569–4598	Fragment 4-1 (4569–5406)
5428R-EV	–	CTGTCGAGGGCGAGCTCCAGCCCGGATTG	5377–5406	
5399F-EV	+	CAATCCGGGGCTGGAGCTTCGCCCTCGACAG	5377–5406	Fragment 4-2 (5377–5851)
5873R-EV	–	TGGAGTTCATGTCAACAGAAGTAGGGGTAG	5822–5851	
5844F-EV	+	CTACCCCTACTCTGTGACATGAACCTCCA	5822–5851	Fragment 4-3 (5822–6185)
6207R-EV	–	GTTCCATCGGCACCGCGCGCAGCCGATG	6157–6185	
6179F-EV	+	CATCGGCTGCGCCGCGGTGCCGATGGAAAC	6157–6185	Fragment 5-1 (6157–7101)
7101R-EV	–	AGTAGACTGGAAAGGCGCAACCTGC	7077–7101	
6981F-EV	+	CTCGGGTGGGTGTGTTAGCTCCACACTCGG	6959–6988	Fragment 5-2 (6959–7266)
SmartIIA-Hind	–	<u>GCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCG</u> <u>GCTTAAGCAGTGGTATCAACGCAGAAAGCTTTTTTTTT</u> TTTTTTTTTTTTTTTTTT ^d	7238–7266	
D81-F	+	ATGTGCCCTAGGGCTGTTCTGTG	5173–5196	D81F/ORF2-52aa-Pac-R
ORF2-52aa-Pac-R	–	<u>AATTAATTAAATTAAGCAAGGCCGAGTGTGGAG</u> ^e	6977–6995	(5173–6995)
ORF2-52aa-del-F	+	TCCACACTCGGCCCTTGCTTAACTTGAGGATACTATTGACTAT ^f	6978–7020	
ORF2-52aa-del-R	–	ATAGTCAATAGTATCTCAAGTTAAGCAAGGCCGAGTGTGGA ^f	6978–7020	D81-F/ORF2-52aa-del-R
7224R	–	AGGGAGCGCGAAAAGCAGAAAAGAAAAAT	7196–7224	ORF2-52aa-del-F/7224R
HEV-G3-ANYF	+	ACCCCGGCAGTTGGTTTT	179–196	HEV-G3-ANYF/ANYR
HEV-G3-ANYR	–	CCCCTGGATAGGATGATTC	212–234	(179–234)
HEV-G3-ANYM1	+	FAM-CGCCCTGAGGTACTT-BHQ-1 ^g	198–212	
83-2-6564F	+	GCTTCGTGCTAATGATGTTCTGTG	6564–6587	83-2-6564F/3'-terminal end
83-2-6940F	+	CACCCAGGCTAGTGGTGTAGGTAGA	6940–6964	83-2-6940F/3'-terminal end
ORF2-R-pacI	–	GAGAATTAAGACTCCCGGGTTTTAC	7136–7160	83-2-6564F/ORF2-R-pacI
				(6564–7160)

^a Polarity of the primer on the HEV genome. +, forward; –, reverse.^b In G3-HEV83-2-27 (GenBank accession no. AB740232).^c The underlined sequence contains the T7 promoter.^d The underlined sequence contains a SmartIIA-specific sequence and a HindIII-digestible sequence.^e The underlined sequence contains a PacI-digestible sequence.^f The mutated nucleotides are underlined.^g The fluorophore 6-carboxyfluorescein (FAM) is attached to the 5' end of the probe, and a quencher, Black Hole Quencher-1 (BHQ-1), is attached to the 3' end.

distinct peaks (F8 and F9) harboring the GUU (Val-encoding) and GAC (Asp-encoding) codons, respectively. These changed RNA sequences were predicted to encode full-length revertant capsid proteins.

Large scale. To clarify the precise growth kinetics of Amut, a larger-scale transfection of Amut RNA was performed. Specifically, the large-scale transfection was performed on a scale approximately 30-fold larger than that described above, and culture supernatants were collected periodically. This procedure permitted a time course of quantification by ELISA analysis and showed that the peak of antigen accumulation occurred 25 days posttransfection, while the number of viral genomes progressively declined during the 2 months of the study (except for small recoveries in copy number on day 25 and at the end of the study) (Fig. 2A). These data suggested the production of a low level of infectious particles from Amut transfection. However, the nonreverted Amut antigens could not be distinguished by WB in the normal- and large-scale experiments, suggesting that the Amut products were unstable,

of low infectivity, and/or produced in small amounts. To confirm the nature of the Amut product, pooled supernatants were subjected to partial purification and SDGA. WB of the resulting fractions detected a 72-kDa band in F7 (specific density, 1.15 g/ml) (Fig. 2B). Quantification of the HEV RNA genome in the fractions detected a single peak, primarily in F7 (Fig. 2C). Determination of the F7 sequence revealed that the codon expected to be an amber codon was instead GUC (complete reversion). Additionally, infection assays demonstrated that F7 readily infected cells (Fig. 2D). Based on our subsequent experiments, we suspect that the end product of the large-scale experiment likely corresponded to a revertant to WT.

Huge scale. To clarify the apparent reversion of Amut, transfection was performed at an even larger scale (10-fold increased over the large scale); culture supernatants were collected periodically, and viral sequences from these samples were determined. The results clearly showed a population shift from the originating amber codon of Amut to the complete revertant (GUC) via an intermediate mutant (GAC) (Table 2). Reversion mutants were

FIG 1 Initial characterization of Amut and WT HEV. (A) Time course of antigen production following transfection with Amut or WT HEV. HEV antigen levels were measured by ELISA using an anti-G3-HEV-VLP rabbit polyclonal antibody. OD₄₉₂, optical density at 492 nm. (B and C) Sedimentation analyses of the Amut product (B) and of the WT product, used as a control (C). Concentrated supernatants derived from 50-ml cultures were sedimented on continuous sucrose gradients (10% to 60% [wt/vol] in phosphate-buffered saline). The resulting fractions were assessed for specific density and the HEV RNA genome copy number (by real-time reverse transcription-PCR). Note the distinct y-axis scales in panels B and C.

not detected until 3 weeks posttransfection. The reproducible reversion of Amut provides evidence of the functional essentiality of the C52aa domain for the HEV life cycle.

To permit analysis of the Amut clone in the absence of revertants, culture supernatants collected within the first 10 days were

FIG 2 Growth kinetics and character of Amut. (A) Supernatants were collected periodically during 2 months of culturing, and HEV antigen levels were measured by ELISA using an anti-G3-HEV-VLP rabbit polyclonal antibody; the HEV RNA genome copy number was determined by real-time reverse transcription-PCR. OD₄₉₂, optical density at 492 nm. Supernatants from a pooled total of 3 liters of culture were concentrated and sedimented. (B) Fractions were subjected to Western blotting using an anti-G3-HEV-VLP rabbit polyclonal antibody. NC, negative control (untransfected cells). P, positive control (HEV-L-VLPs). 75 K, 75,000 (molecular weight). Symbols designate the positions of the major band in the Amut supernatant (open arrowhead) and the HEV-L-VLP (filled arrowhead). (C) Fractions were assessed for the HEV RNA genome copy number and specific density. (D) Confirmation of the infectivity of fraction 7 by ELISA.

pooled and subjected to partial purification and SDGA. WB detected multiple bands of approximately 55 kDa and smaller, starting in F7; these bands formed a broad range, with peak accumulation detected in F10 (specific density, 1.21 g/ml) (Fig. 3A). In contrast, F8 (specific density, 1.15 g/ml) had the highest copy number of the genome (Fig. 3B). For subsequent analysis, F8 and F10 were designated the minor and major products (Mip and Map, respectively) based on antigen levels. To determine the RNase sensitivities of the products, the fractions were treated with 20 μ g/ml of RNase A for 30 min at 37°C. The RNase resistance of the fractions was confirmed by RT-PCR quantification analysis, indicating viral encapsidation. Both products exhibited resistance to RNase treatment (Fig. 3C), indicating the presence of encapsidated RNA. Neither the GAC nor the GUC reversion mutation was detected in these products by RT-PCR sequencing analysis, suggesting that those specific alleles were largely absent from this population.

Further analysis of peak discrepancy between the antigen level and the genome copy number revealed two points. First, the copy

TABLE 2 Time course sequence of the codon mutated to an amber codon for the supernatants of Amut-transfected cells

Codon	Sequence ^a at the following day posttransfection:								
	7	10	14	17	21	24	28	31	35
Amber mutant	UAA	UAA	UAA	UAA	UAA	ND	ND	ND	ND
Revertant									
Intermediate	ND	ND	ND	ND	ND	GAC	GAC	ND	ND
Complete	ND	ND	ND	ND	ND	GUC	GUC	GUC	GUC

^a Determined for the first codon of the C52aa-encoding region of the ORF2 gene. ND, not detected.

number in the Map fraction was approximately 15 times lower than that in the Mip fraction (Fig. 3B). Second, the constitution (genome/antigen) ratio in the Map fraction was approximately 40-fold lower than that in the Mip fraction by analysis using Image

Gauge, version 4.0 (Fujifilm, Tokyo, Japan); the ratio in the Mip fraction was approximately equal to that of WT (Fig. 3D). On the other hand, the RNA content of the Map fraction was extremely reduced, suggesting that these products represented empty parti-

FIG 3 Encapsulation of the Amut genome and its characteristics. (A) Fractions were subjected to Western blotting using an anti-G3-HEV-VLP rabbit polyclonal antibody. NC, negative control (uninfected cells). Symbols indicate the positions of the major bands in the Amut fraction (55 kDa) (open arrowhead) and the WT fraction, used as a positive (P) control (72 kDa) (filled arrowhead). (B) Fractions were assessed for the HEV RNA genome copy number and specific density. (C) RNase resistance was measured as the ratio of the level of HEV RNA in RNase-treated fractions to that in untreated fractions (HEV RNA reduction ratio). WT virions and extracted WT RNA were used as positive and negative controls, respectively. (D) Constitution (genome/antigen) ratios (actual values are shown above the bars) were calculated by dividing the genome quantities from panel B by the chemiluminescence intensities from panel A. (E) To confirm the infectivity of the indicated fractions, cells were inoculated and periodically analyzed by ELISA using an anti-G3-HEV-VLP rabbit polyclonal antibody. OD₄₉₂, optical density at 492 nm.

FIG 4 Detection of degraded capsid termini in Amut. HEV small virus-like particles (HEV-S-VLP), HEV large virus-like particles (HEV-L-VLP), and fraction 9 (derived as described for Fig. 3A) were stained with Coomassie brilliant blue (CBB) or were subjected to Western blot analysis using a monoclonal antibody specific to both HEV-S-VLP and HEV-L-VLP (MAb 53) or to HEV-L-VLP alone (MAb 68). Lane M, molecular weight markers.

cles; this inference is consistent with the low productivity of Amut products on all scales. Specifically, we observed that the Map fraction could not infect cells (Fig. 3E), while the Mip fraction was infectious for these cells (Fig. 3E) and yielded reversion mutants (GUC) during long-term observation (data not shown). While the viral reproduction of Amut was impaired, the Mip fraction could sustain low levels of viral production, leading to the emergence of revertants as shown in the large-scale experiment (Fig. 2A).

The observation, via WB (Fig. 3A), of a “smear” of antigen with a maximum size of 55 kDa was unexpected, given that the capsid protein lacking C52aa (predicted size, 6 kDa) was expected to migrate at 66 kDa (that is, 72 kDa less 6 kDa). The observed 11-kDa decrease in size suggested further degradation of the capsid in the absence of the C52aa domain. Mass spectrometry followed by protein sequencing detected two fragments with amino acid sequences corresponding to early N-terminal capsid sequences. The presence of the capsid N-terminal domain was confirmed by detection with monoclonal antibody (MAb) 68 (Fig. 4), a reagent that exhibits specificity for HEV-L-VLP (specific to the N-terminal 13 to 111 aa) (T. C. Li, unpublished observations). In contrast, the protein was not detected using the HEV-S-VLP- and HEV-L-VLP-specific MAb 53 (Fig. 4), implying the absence of the S-and-L common region. Protein sequencing and reactivity with the HEV-VLP-specific antibodies strongly suggested that the 55-kDa bands correspond to proteolytic products generated by degradation from the C terminus on the viral surface, presumably via loss of the P domain. Further degradation (to lower-molecular-weight species) probably occurred after encapsidation, given that previous studies showed that this region was essential for dimerization and particle formation by the capsid (3, 15, 16).

HEV virions exhibit distinct buoyant densities in feces (1.26 to 1.27 g/ml) and in circulating blood (1.15 to 1.16 g/ml), differences that might be associated with their cellular membrane content (17). The density of the Amut Map fraction was higher than that of the Mip fraction. This result is inconsistent with the notion that the Map is an empty particle (18). The Amut Mip fraction had the specific density of membrane-associated virions, although the ORF3 (egress-related) protein was not detected in these particles, in contrast to WT particles (T. Shiota, unpublished observations) (19). We hypothesize that the correct encapsidation of Amut resulted in an enveloped particle lacking the ORF3 protein (Mip; density, 1.15 g/ml), whereas the incorrect encapsidation of Amut resulted in a nonenveloped and

(usually) empty particle (Map; density, 1.21 g/ml), the density of which was intermediate between that of the membrane-associated virion (1.15 to 1.16 g/ml) and the nonenveloped filled virion (1.26 to 1.27 g/ml) (17).

In the present study, we showed that the C52aa domain of the HEV capsid was essential for the HEV life cycle, as confirmed by reproducible reversion at the amber mutation, which would otherwise truncate the C52aa domain. The presence of the C52aa domain promoted the accurate encapsidation of HEV and protected the particle from further C-terminal degradation. To clarify the involvement of the C52aa domain in neutralization, further studies (e.g., using a MAb specific for this region) will be required.

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