Virus Research 161 (2011) 65–77

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Review

Hepatitis E virus cell culture models

Hiroaki Okamoto*

Division of Virology, Department of Infection and Immunity, Jichi Medical University, School of Medicine, Tochigi-Ken 329-0498, Japan

ARTICLE INFO

Article history:
Available online 21 February 2011

Keywords:
Hepatitis E virus
Cell culture
Genotype
Infectious clone

ABSTRACT

Early studies reported the propagation of hepatitis E virus (HEV) in either primary hepatocytes or several established cell lines, but replication was inefficient. Efficient cell culture systems for HEV in PLC/PRF/5 and A549 cells have recently been established, using inoculum of fecal suspensions with high HEV loads, originally obtained from patients with genotype 3 HEV (the JE03-1760F strain, 2.0×10^7 copies/ml) or genotype 4 HEV (the HE-JF5/15F strain, 1.3×10^7 copies/ml), and many generations were successfully propagated in serial passages of culture supernatant. In addition, a full-length infectious cDNA clone (pJE03-1760F/wt) of the JE03-1760F strain was constructed, which can replicate efficiently in PLC/PRF/5 and A549 cells. A derivative ORF3-deficient mutant revealed that the ORF3 protein of HEV is responsible for virion egress from infected cells and is present on the surface of released HEV particles, which is associated with lipids. Various HEV strains with high loads of $\geq 10^5$ copies/ml in circulating blood were also propagated efficiently in PLC/PRF/5 and A549 cells. This paper reviews the road map toward the development of efficient cell culture systems for a wide variety of HEV strains and introduces the current knowledge on virion egress obtained by cell culture models.

Contents

1. Introduction

Hepatitis E virus (HEV) was discovered in 1983 by immune electron microscopy (Balayan et al., 1983) and was first cloned in 1990 (Reyes et al., 1990). HEV is the causative agent of acute or fulminant hepatitis E, which occurs in many parts of the world, principally as a water-borne infection in developing countries and zoonotically in industrialized countries (Chandra et al., 2008; Colson et al., 2010; Dalton et al., 2008; Emerson and Purcell, 2007; Purcell and Emerson, 2008; Tei et al., 2003; Yazaki et al., 2003). Several species of animals, such as swine, wild boars and deer, are considered to serve as reservoirs of HEV (Meng et al., 1997b; Pavio et al., 2010; Reuter et al., 2009; Sonoda et al., 2004; Takahashi et al., 2003, 2004a), and ingestion of uncooked or undercooked meat and the viscera of these animals may be the major route of HEV infection in industrialized countries, including Japan (Tei et al., 2003; Yazaki et al., 2003). HEV was previously classified in the family *Caliciviridae*, but it is now placed in the genus *Hepevirus* within the family *Hepeviridae* (Emerson et al., 2005a). The virion is 27–34 nm in diameter and is believed to be non-enveloped, although HEV particles in the culture supernatant and circulating blood are associated with lipids, as described in detail below. Its genome consists of a single-stranded, positive-sense RNA of approximately 7.2 kilobases (kb) in length, which is capped and polyadenylated (Kabrane-Lazizi et al., 1999; Tam et al., 1991). It contains a short 5′ untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3), and 3′UTR. ORF1 encodes non-structural proteins including methyltransferase, papain-like cysteine protease, helicase and

* Tel.: +81 285 58 7404; fax: +81 285 44 1557.
E-mail address: hokamoto@jichi.ac.jp

0168-1702/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2011.01.015

RNA-dependent RNA polymerase (Agrawal et al., 2001; Koonin et al., 1992). ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA of 2.2-kb in length (Graff et al., 2006). The ORF2 protein is the viral capsid protein; the crystal structure of a truncated recombinant ORF2 protein has been elucidated, but the size of the protein in mature virions remains unknown (Guu et al., 2009; Yamashita et al., 2009). The ORF3 protein is a small, phosphorylated protein made of 113 or 114 amino acids (aa), whose function(s) has not been fully defined (Emerson et al., 2010). Four genotypes of HEV that infect humans have been identified (Lu et al., 2006; Okamoto, 2007). Genotypes 1 and 2 were isolated from humans only and are mainly seen in developing countries. Genotypes 3 and 4 are zoonotic, and have been identified in many sporadic cases affecting middle-aged and elderly men in industrialized countries (Lewis et al., 2010; Meng, 2010; Okamoto et al., 2003; Purcell and Emerson, 2008).

HEV transmission studies have mostly been done in nonhuman primates such as macaque species, chimpanzees, and owl monkeys, which have provided important information regarding the biology and pathogenesis of HEV (Purcell and Emerson, 2001). Experimental transmission studies have also been done in pigs, an established reservoir for HEV (Williams et al., 2001). In the swine model, replicative, negative-strand HEV RNA was detected primarily in the small intestines, lymph nodes, colon, and livers, indicating that HEV replicates in tissues other than the liver. The establishment of a practical cell culture system that facilitates the propagation of HEV *in vitro* is critical for virological characterization as well as for studies on the prevention of HEV infection. The propagation and production of HEV *in vitro* have been attempted by many researchers in primary hepatocytes from nonhuman primates (Chimpanzees, cynomolgus macaques, tamarins, and African green monkeys) (Arankalle et al., 1988; Kane et al., 1984; Tam et al., 1996, 1997) and in various continuous cell lines, such as the human normal embryonic liver cells (WRL68), human hepatoma cell lines (PLC/PRF/5, HepG2, and Huh-7 cells), human colon carcinoma cells (Caco-2), human embryo lung diploid cells (2BS), human lung embryonal fibroblast cells (MRC-5), human lung cancer cells (A549), human choriocarcinoma cells (HCCM), African green monkey kidney cells (Vero), and Rhesus monkey kidney from *Macaca mullata* cells (LLC-MK2) (Arankalle et al., 1988; Emerson et al., 2010; Huang et al., 1992, 1995, 1999; Kazachkov Yu et al., 1992; Li et al., 1996; Meng et al., 1996, 1997a; Wei et al., 2000). In addition, Emerson et al. (2005b, 2006a) reported a quantitative cell culture assay for performing both neutralization tests and thermal stability tests by detecting infected HepG2/C3A cells by immunofluorescence microscopy. However, none of these culture systems can provide high-titer infectious HEV in the culture supernatant. This laboratory recently succeeded in establishing efficient cell culture systems for infectious HEV in feces and circulating blood, which has opened up new avenues for analyzing the virus itself that have not been possible since the discovery of the viruses by Balayan et al. (1983) in 1983. This article reviews the establishment and evaluation of cell culture systems for various HEV strains, and summarizes the new knowledge that has evolved by the HEV cell culture models.

2. Propagation of infectious HEV strains of genotypes 3 and 4 in feces and serial passages of their progenies in cultured cells

Each of 21 selected cell lines was inoculated with fecal suspensions with high HEV load (JE03-1760F strain: 2.0×10^7 copies/ml) obtained from a Japanese patient who contracted a domestic infection of genotype 3 HEV. The cell lines were derived from humans [PLC/PRF/5 (CRL-8024), HepG2 (HB-8065), Huh-7 (RCB1366), A549 (CCL-185), MCF (JCRB0314), NUGU-4 (JCRB0834), HEK293 (CRL-1573), HeLa (CCL-13), HT-1080 (JCRB9113), SK-N-MC (HTB-10), and GOTO (JCRB0612)], mice [P19 (CRL-1825), BC3H1 (CRL-1443), L929 (RCB1422), and C2C12 (CRL-1772)], rats [C6 (CCL-107), and IEC-6 (CRL-1592)], monkeys [LLC-MK2 (CCL-7), and CV1 (CRL-10478)], cow [MDBK (CCL-22)], and dog [MDCK (CCL-34)], and were available from either the American Type Culture Collection (ATCC, Manassas, VA) or the RIKEN BRC Cell Bank (RIKEN BioResource Center, Tsukuba, Japan) (Tanaka et al., 2007). Monolayers of cultured cells in a six-well microplate were inoculated with 0.2 ml of the virus stock that had been diluted with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)], and containing 0.2% (w/v) bovine serum albumin, and filtered. After having been inoculated for 1 h at room temperature, the solution was removed and 2 ml of maintenance medium was added and cultured at 35.5 °C in a humidified 5% CO_2 atmosphere. The maintenance medium used for virus culturing was 50% Dulbecco's Modified Eagle Medium (DMEM, GIBCO Cat. No. 12800-017, Invitrogen, Tokyo, Japan) and 50% Medium 199 (GIBCO Cat. No. 31100-027, Invitrogen) containing 2% (v/v) heat-inactivated fetal bovine serum and 30 mM $MgCl_2$ at final concentration. One-half (1 ml) of the culture medium was replaced with fresh maintenance medium every other day since day 2 after inoculation, and the harvested media were stored at −80 °C until testing. The JE03-1760F strain was found to replicate efficiently in two of the 21 cell lines tested; PLC/PRF/5 from human hepatocellular carcinoma and A549 from human lung cancer. HEV progenies were released into culture supernatant in titers reflective of the HEV inoculum. Cultures were inoculated with 1.0×10^5 copies [multiplicity of infection (MOI): approximately 0.1] per well in a six-well plate of PLC/PRF/5 cells, and HEV was first detectable in the culture supernatant 8 days post-inoculation (dpi) and reached 10^8 copies/ml on 50 dpi in all six wells (Fig. 1A). HEV was detectable in culture media of all six wells even when the inoculum size was reduced to 1.0×10^4 copies (MOI: approximately 0.01) per well, although the initial day of appearance was later and highest titer of HEV in the culture supernatant after inoculation decreased. HEV RNA was detectable in only one of the six wells inoculated following inoculation at 1.0×10^3 copies (MOI: approximately 0.001), with less efficient multiplication. A549 cells were seeded at 1.0×10^4 and 1.0×10^5 copies per well, and HEV RNA reached the highest titer of 10^7 copies/ml on 50 dpi in all wells, independent on the titer of seed virus in the inoculum, although it was initially detected on 6 and 4 dpi, respectively. HEV RNA was detectable in two of the six wells inoculated at 1.0×10^3 copies per well, reaching the titer of 10^6 copies/ml on 50 dpi. These results suggest that the highest HEV RNA titer in the culture supernatant during cell culture and the interval between inoculation of seed virus and the appearance of progenies in the culture supernatant differ depending on the cell type inoculated, even when inoculated at the same MOI. Inoculation at higher MOI allows PLC/PRF/5 cells to support replication of JE03-1760F HEV at higher efficiency than A549 cells. On the other hand, A549 cells can support more efficient growth than PLC/PRF/5 cells when inoculated at lower MOI, with an earlier appearance of HEV progenies in the culture medium.

The ORF2 protein in PLC/PRF/5 cells was stained by an immunofluorescence assay (IFA) with a mouse monoclonal antibody against ORF2 protein (anti-ORF2 mAb) (H6225) (Takahashi et al., 2008a). The cultured cells that showed positive staining for ORF2 protein increased over time, thus reflecting a spread in HEV infection in these cells (Fig. 1B). Previous reports suggested that HEV can be cultured in PLC/PRF/5 and A549 cells, although the viral titers in culture media are extremely low, and virus spreading in cultured cells was not seen in any of previously reported cell culture systems for HEV (Huang et al., 1992; Li et al., 1996; Meng et al., 1996, 1997a; Pillot et al., 1987). The first successful propagation of the JE03-1760F strain in PLC/PRF/5 and A549 cells may have been

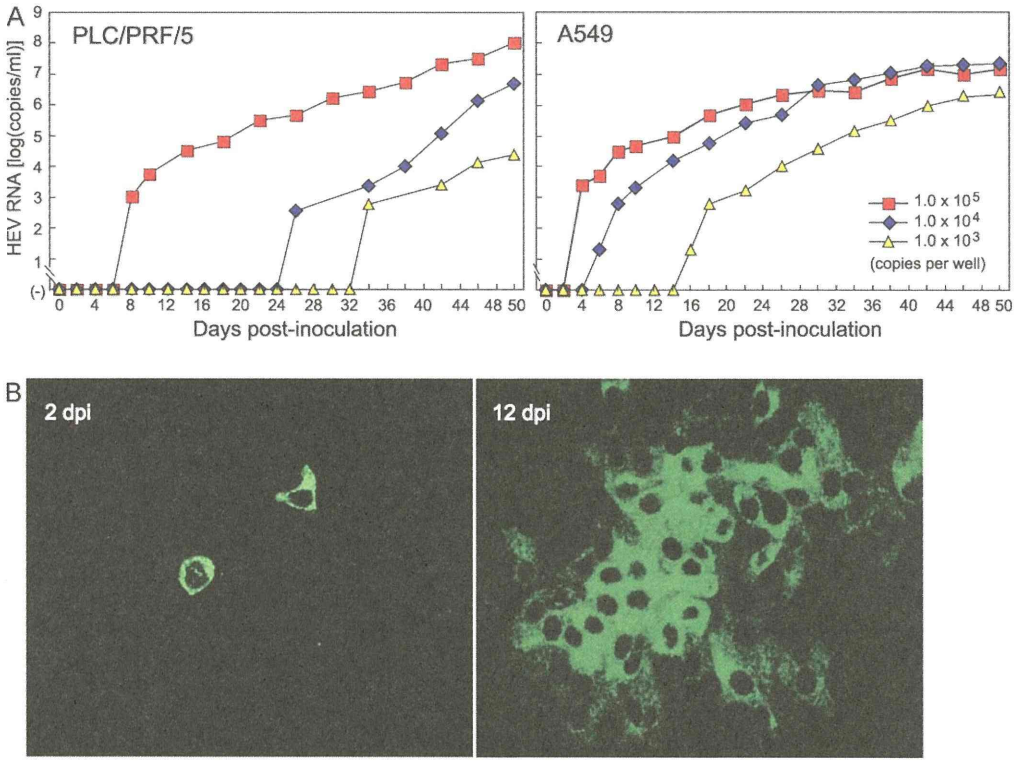


Fig. 1. Replication of HEV in PLC/PRF/5 and A549 cells. (A) Quantification of HEV RNA in culture supernatants of PLC/PRF/5 or A549 cells after inoculation with diluted fecal supernatant (JE03-1760F) containing the indicated HEV loads. (B) Immunofluorescent staining of the JE03-1760F HEV-inoculated PLC/PRF/5 cells by an anti-ORF2 mAb (H6225) and Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) on 2 and 12 dpi. Images of stained cells were obtained using a BZ-8000 digital microscope (Keyence, Osaka, Japan).

ascribable to the markedly high HEV titer in the inoculum and the 29 point mutations with 6 non-synonymous mutations found in the virus in feces (DDBJ/EMBL/GenBank accession no. AB301710), which were not possessed by any of reported genotype 3 HEV strains (Takahashi et al., 2007).

Progenies of the genotype 3 JE03-1760F strain released in the culture medium were passaged 10 times in PLC/PRF/5 cells at an inoculum titer of 3.6×10^4 to 5.1×10^6 (median, 9.8×10^4) copies per well (Experiment A; Fig. 2A) (Lorenzo et al., 2008). HEV RNA increased to 6.4×10^5 copies/ml on 28 dpi in the primary propagation (Passage 0). Probably due to the low viral load of the inoculum (3.6×10^4 copies per well), HEV RNA in the first passage (Passage 1) was not detectable over 5 weeks in the harvested culture medium, but it appeared on 36 dpi and reached a maximum load of 8.6×10^6 copies/ml on 56 dpi. In Passages 2–5, HEV RNA appeared on 12–16 dpi, and thereafter grew in a manner similar to those in Passage 0. In Passages 6–10, HEV RNA was first detectable in the culture medium on 8, 10, or 12 (mean, 10.0) dpi, and reached a load of $>10^5$ copies/ml on 12–22 (mean, 16.0) dpi, in contrast to Passages 0–5 where HEV RNA appeared on 10–36 (mean, 16.3) dpi and HEV load in the culture supernatant became $>10^5$ copies/ml on 24–48 (mean, 35.2) dpi (Fig. 2A).

In an additional experiment (Experiment B in Fig. 2B), 10 generations of serial passages (Passages 1–5 in PLC/PRF/5 and Passages 6–10 in A549) were performed after primary propagation of the JE03-1760F strain in PLC/PRF/5 cells for 56 days. A shorter duration before the appearance of HEV in culture medium was also achieved after Passages 6–10 in comparison to Passages 0–5. Eventually, HEV became detectable after only 2 days following the inoculation in culture medium for Passages 5–10, with the time before virus titers reached 10^5 copies/ml shortened by an average of 1 week (17.0 vs. 9.6 days), and the titer of HEV in culture medium exceeded 10^6 copies/ml on 10 dpi of Passage 10 (Fig. 2B).

The initial success on the development of a cell culture system for the genotype 3 HEV JE03-1760F strain led to the discovery that the HE-JF5/15F strain of genotype 4 in a fecal suspension with a high HEV load of 1.3×10^7 copies/ml that was recovered from a Japanese patient with fulminant hepatitis E, can also replicate efficiently in PLC/PRF/5 cells (Tanaka et al., 2009). HEV RNA appeared in the culture media of PLC/PRF/5 cells on 16 dpi in the primary propagation (Passage 0), with a viral load of 1.3×10^3 copies/ml, and continued to increase thereafter, with a maximum load of 2.8×10^6 copies/ml on 60 dpi (Fig. 2C). HEV progenies released in the culture medium of PLC/PRF/5 cells were passaged six times in PLC/PRF/5 or A549 cells. HEV RNA in the first passage (Passage 1) appeared on 6 dpi and reached a maximum load of 5.0×10^6 copies/ml on 42 dpi in the culture medium of PLC/PRF/5 cells. HEV progenies in Passage 1 were inoculated on PLC/PRF/5 and A549 cells, at a load of 5.0×10^5 copies per well, and A549 cells supported more efficient multiplication of HEV than PLC/PRF/5 cells. Therefore, A549 cells were used as host cells for virus culturing in place of PLC/PRF/5 cells in subsequent passages (Passages 2–6). The HEV progenies in Passage 6 (HE-JF5/15F_p6) grew efficiently, with a high HEV load of 1.5×10^8–3.9×10^8 copies/ml in the harvested culture medium at 10–20 dpi (Fig. 2C). A Western blot analysis using anti-ORF2 mAb (H6210) revealed a single 83-kDa band representing ORF2 protein was detected in 10 μl of the culture supernatant of Passage 6 at 10–20 dpi (Fig. 2D). Shortening of the interval between inoculation of cultures and maximum virus yield as well as increases in the yield of virus indicate adaptation to growth in cell culture. All six wells (100%) showed persistent virus appearance following inoculation of wild-type HE-JF5/15F at 1.0×10^5 copies per well. On the other hand, inoculation with HEV at 1.0×10^4 copies per well yielded culture supernatants that were negative for HEV RNA in all six wells for wild-type virus throughout the observation period of 50 days. In contrast, inoculation with HE-JF5/15F_p6 HEV

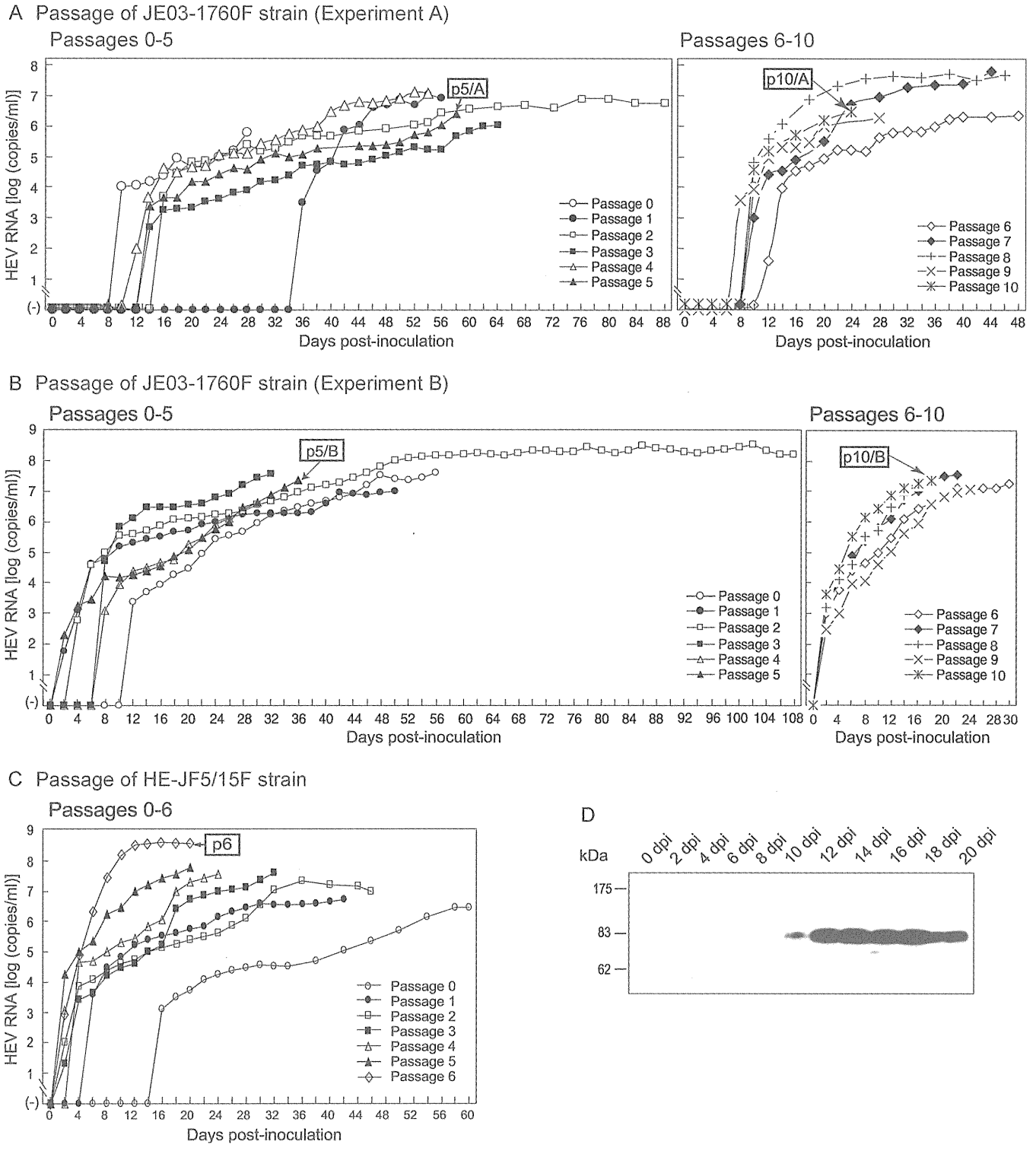


Fig. 2. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 or A549 cells inoculated with fecal supernatant or culture medium of JE03-1760F strain in Experiment A (A) or Experiment B (B), or fecal supernatant or culture medium of HE-JF5/15F strain (C), that was harvested on the final day of each passage. The boxed letters of p5/A and p10/A in (A), p5/B and p10/B in (B), and p6 in (C) denote the days after inoculation when culture supernatants were harvested for molecular cloning of the HEV genomes. (D) A Western blot analysis of ORF2 protein in the culture supernatant. ORF2 protein in culture supernatant was detectable by Western blotting with an anti-ORF2 mAb (H6210) on 10–20 dpi of Passage 6.
Modified from Lorenzo et al. (2008) and Tanaka et al. (2009).

at 3.0×10^3 copies per well, yielded continuous growth in all six wells (100%); and in two (33%) of six wells with an inoculation of HEV at 1.0×10^3 copies per well. In addition, even upon inoculation of a lower dose of 1.0×10^3 copies per well, HE-JF5/15_p6 showed earlier appearance of progenies with an HEV load of $>10^4$ copies/ml in the culture supernatant than the wild-type which had been inoculated at 1.0×10^5 copies per well (26.0 dpi vs. 35.0 dpi), indicating that the wild-type HE-JF5/15F HEV adapted to growth in cell culture during 6 consecutive passages in this cell culture system.

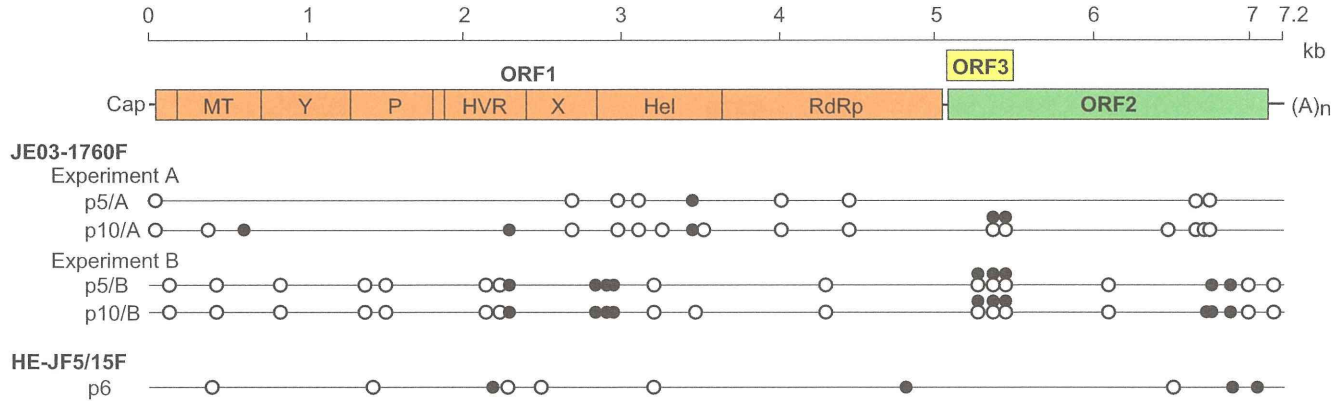


Fig. 3. Comparison of the sequence of wild-type JE03-1760F and its cell culture-generated variants (p5/A, p10/A, p5/B, and p10/B) as well as wild-type HE-JF5/15F and its cell culture-produced variant p6 over the entire genome. (Upper panel) The genomic structure of HEV. Abbreviations are: MT, methyltransferase; Y, Y domain; P, papain-like protease; HVR, hypervariable region; X, X domain; Hel, helicase; and RdRp, RNA-dependent RNA polymerase. (Lower panel) Open circles denote synonymous substitutions and closed circles depict non-synonymous substitutions. See Table 1 for a comparison of the accession numbers of HEV isolates.

Furthermore, HEV progenies of genotype 3 JE03-1760F strain in the culture supernatant replicated efficiently through many passages in PLC/PRF/5 and A549 cells (45 passages as of October 2010), with the highest HEV RNA titer of 10^8–10^9 copies/ml in the culture media (Unpublished observations). In addition, HEV progenies of the genotype 4 HE-JF5/15F strain in the culture supernatant grew efficiently through many passages in PLC/PRF/5 and A549 cells (25 passages as of October 2010), with the highest HEV loads of 10^9–10^{10} copies/ml in the culture supernatants (Unpublished observations). No cytopathic effect (CPE) was observed in the PLC/PRF/5 and A549 cells during these serial passages of the JE03-1760F and HE-JF5/15F strains, despite differences in the duration of cell culture and the changing profile of HEV load in each passage.

3. Mutational events during cell culture of infectious HEV strains

The entire genomic sequences of Passage 5 (p5/A) and Passage 10 (p10/A) strains in Experiment A and Passage 5 (p5/B) and Passage 10 (p10/B) strains in Experiment B (see Fig. 2) were determined and compared with that of the parent wild-type JE03-1760F HEV to identify the mechanism of adaptation during cell culture of the JE03-1760F strain (Fig. 3). They all had the same genomic length of 7226 nucleotides (nt) as the wild-type JE03-1760F [the poly(A) tract at the 3′-terminus excluded]. Few genetic changes (0.3%) were found in HEV progenies thriving in cell culture similar to the adaptation of hepatitis A virus (HAV), another enterically transmitted hepatitis virus (Graff et al., 1994; Hu et al., 2002; Jansen et al., 1988). Briefly, the p5/A isolate possessed 9 mutations which were restricted to 5′UTR, ORF1 or ORF2. In comparison to the p5/A isolate, 9 additional mutations were present in ORF1, ORF2, or the ORF2/ORF3 overlapping region of the p10/A isolate, which differed by 18 nt from the wild-type over the entire genome (Table 1). In addition to the mutation at nt 3453 which first appeared in ORF1 (1143 aa) of the p5/A isolate, 4 mutations occurred in the p10/A isolate at nt 591, 2246, 5378, and 5456 resulting in amino acid changes in ORF1 (aa 189 and 741) or ORF3 (aa 73 and 99). Of interest, the p5/B isolate in Experiment B differed by 21 nt from the wild-type over the entire genome, including the nucleotide substitutions at nt 2246, 5378, and 5456 that were commonly seen in the p10/A isolate in another series of passages (Experiment A), which may be implicated in the heightened multiplication.

The HE-JF5/15F_p6 isolate had the same genomic length of 7239 nt as the wild-type HEV [the poly(A) tract at the 3′-terminus excluded]. A comparison of mutations over the entire genome and the predicted amino acid differences within the three ORFs between the wild-type HE-JF5/15F and its variant p6 (see Fig. 2C) revealed the p6 isolate harbored 10 mutations that were restricted to ORF1 and ORF2 (Table 1), including a mutation at nt 6549 with the mixed nucleotide population of C as well as the U that the wild-type possessed. There were five mutations at nt 1450, 2518, 4874, 6881, and 7144 in p6 that were not seen in any of the 54 reported genotype 4 HEV isolates whose entire or nearly entire sequence is available from the DDBJ/EMBL/GenBank databases as of October 2010, three of which changed amino acids (aa 1617 in ORF1 and aa 565 and 653 in ORF2). The p6 HEV genome had 5 synonymous and 2 non-synonymous substitutions in ORF1 and one synonymous and 2 non-synonymous substitutions in ORF2, unaccompanied by any substitutions in the overlapping ORF3 (Fig. 3), which was quite dif-

Table 1
Comparison of the cell culture-generated variants with the wild-type HEV isolate.

Feature	JE03-1760F (genotype 3)				HE-JF5/15F (genotype 4)
	p5/A [AB362842]	p5/B [AB425831]	p10/A [AB362843]	p10/B [AB593690]	p6 [AB480825]
HEV RNA titer (copies/ml)	2.4×10^6	2.2×10^7	3.0×10^6	2.2×10^7	3.6×10^8
Days after the initial inoculation	348	316	540	424	244
Nucleotide substitution					
Entire genome (7226 or 7239 nt)	9	21	18	23	10
5′UTR (25 nt)	1	0	1	0	0
ORF1 (5109 or 5121nt) [1703 or 1707 aa]	6 (1)	13 (4)	11 (3)	14 (4)	7 (2)
ORF2 (1980 nt) [660 aa]	2 (0)	7 (2)	6 (0)	8 (3)	3 (2)
ORF3 (339 or 342 nt) [113 or 114 aa]	0	3 (3)	2 (2)	3 (3)	0
3′UTR (75 or 72 nt)	0	1	0	1	0
Mutation rate ($\times 10^{-3}$ nucleotide substitutions per site per year)	1.31	3.36	1.68	2.74	2.04

Modified from Lorenzo et al. (2008) and Tanaka et al. (2009).

Table 2
Reactivity of anti-ORF2 mAb or anti-ORF3 mAb with the HEV particles treated with various reagents, and evaluated by immuno-capture RT-PCR.

Treatment	Percentage of captured HEV in total HEV per well							
	Feces		Culture supernatant (wild-type)		Culture supernatant (ΔORF3 mutant)		Serum	
	Anti-ORF2 mAb (H6225)	Anti-ORF3 mAb (TA0536)	Anti-ORF2 mAb (H6225)	Anti-ORF3 mAb (TA0536)	Anti-ORF2 mAb (H6225)	Anti-ORF3 mAb (TA0536)	Anti-ORF2 mAb (H6225)	Anti-ORF3 mAb (TA0536)
None	93.1	0.0	6.3	5.7	95.7	0.1	1.0	1.5
Tween 20	92.2	0.0	22.1	16.5	95.4	0.0	38.5	37.6
0.2%	95.7	0.4	69.0	76.8	95.6	0.1	55.7	90.4
5%								
10% chloroform								
25 °C, 10 min	92.0	0.1	87.3	54.4	98.0	0.0	28.4	64.7
37 °C, 30 min	99.2	0.0	62.9	9.5	99.0	0.1	35.7	21.5
NP-40 + 2-ME + pronase E[a]	99.9	1.3	98.7	0.2	99.5	0.0	86.3	0.0
NP-40 + 2-ME[a]	99.8	0.4	97.2	77.9	99.2	0.2	78.3	84.2
NP-40 + pronase E[a]	99.4	0.0	99.0	0.2	97.7	0.0	90.4	0.6

Modified from Takahashi et al. (2010) and Yamada et al. (2009a).
[a] Treated with 0.1% NP-40 and/or 0.1% 2-mercaptoethanol (2-ME) and/or 0.1% pronase E.

ferent from the p10/A and p10/B variants of the JE03-1760F strain which had two and three non-synonymous mutations, respectively, in ORF3. Of note, one of the 2 non-synonymous mutations within ORF1 of p6 mapped to the RNA-dependent RNA polymerase region (aa 1617), and may be associated with the heightened replication of p6 toward adaptation in culture. Two putative stem-loop structures comprising nt 7089–7163 and 7173–7194, respectively, at the 3′ end of the HEV genome are important in concert for binding to RNA-dependent RNA polymerase (Agrawal et al., 2001). An A-to-G mutation at nt 7144 might be associated with a heightened replicative activity. Mutations that affect the secondary structures in the 3′-terminal region can influence RNA replication (Graff et al., 2005a). Therefore, synonymous mutations observed in the cell culture-generated variants may also affect viral replication and protein expression. Further studies using the reverse genetics system with *in vitro* mutagenesis techniques are therefore required to clarify whether the observed mutations and those that will be found in extended consecutive passages in cell culture have any drastic effects on the rate and efficiency of growth of the HEV strains during propagation on cell cultures.

It is not possible to estimate the evolutionary rate of HEV by analyzing serial samples collected from an individual host because HEV infection in humans and animals is usually transient. Based on the assumption that HEV can persist in the community by successive transmission from host to host, the mutation rate of HEV has been estimated to be approximately $1.4–1.7 \times 10^{-3}$ nucleotide substitutions per site per year (Takahashi et al., 2004b). The persistent infection during long-term consecutive passages of culture supernatant yielded an estimated mutation rate of HEV of $1.3–3.4 \times 10^{-3}$ nucleotide substitutions per site per year in the JE03-1760F strain and a comparable rate of 2.0×10^{-3} nucleotide substitutions per site per year in HE-JF5/15F strain (Table 2).

4. Construction of an infectious HEV cDNA clone

A full-length infectious cDNA clone (pJE03-1760F/wt) of the genotype 3 JE03-1760F strain was developed (Yamada et al., 2009b). In addition, as a negative control, a frameshift mutant of ORF1, pJE03-1760F/ΔORF1, was constructed after digestion of the pJE03-1760F/wt plasmid DNA with *Aat*II, followed by blunting with T4 DNA polymerase and self-ligation (Fig. 4A). To evaluate the reverse genetics system for HEV using the pJE03-1760F/wt in cell culture, HEV RNA in culture supernatants of RNA-transfected PLC/PRF/5 cells were quantified by measuring the RNA titer in the culture supernatant by real-time RT-PCR. The HEV RNA titer decreased to approximately 10^6 copies/ml in all samples tested on 4 days post-transfection (dpt), because of the medium change on 2 dpt (Fig. 4B). Thereafter, a gradual decrease in HEV load was observed in the culture medium of the ΔORF1 mutant RNA-transfected cells, whose titer likely reflected residual amounts of the introduced RNA transcripts in the culture medium. On the other hand, the viral RNA levels in the pJE03-1760F/wt RNA-transfected cells started to increase on 8 dpt and reached greater than 10^7 copies/ml on 28 dpt, and was maintained on the order of 10^7 copies/ml in the pJE03-1760F/wt transfection up through 60 dpt. ORF2 protein expression was detectable by IFA with anti-ORF2 mAb (H6225) in the transfected cells on ≥5 dpt, with increasing levels of ORF2 antigens, suggesting the spread of HEV infection in cell culture (Yamada et al., 2009b). A Western blot analysis with anti-ORF2 mAb (H6210) revealed a single 83-kDa band in the culture medium of the pJE03-1760F/wt RNA transfection (lane 1, Fig. 4C) but not that of ΔORF1 mutant RNA transfection (lane 2). The 83-kDa single band was also detected in the lysate of the pJE03-1760F/wt RNA-transfected cells (lane 5), but not in the lysate of ΔORF1 mutant RNA-transfected cells (lane 6) nor mock-transfected cells (lane 4). For comparison purposes, an expression plasmid of ORF2 protein of JE03-1760F strain, pCI-HEVORF2 containing the full-length ORF2 sequence of the JE03-1760F genome, was transfected into PLC/PRF/5 cells. Interestingly, the molecular mass of ORF2 protein expressed by the pCI expression plasmid (lane 3) was slightly less than that detected in the pJE03-1760F/wt RNA-transfected cells. The glycosylated form of ORF2 protein gradually shifts to the non-glycosylated form in the cytoplasm through the retrotranslocation pathway (Surjit et al., 2007), and only the non-glycosylated form in the cytoplasm is stable in the cytoplasm of mammalian cells (Torresi et al., 1999). However, the ORF2 protein of 83 kDa detected in the culture supernatants and lysates of the pJE03-1760F/wt RNA-transfected cells (Fig. 4C) and culture supernatant of cells infected with cell culture-generated HEV (Fig. 2D) was likely to be glycosylated. Graff et al. (2008) recently reported that the formation of infectious virus particles was prevented by mutations within the potential glycosylation sites in the ORF2 protein. Taken together, the glycosylated form of ORF2 protein is likely to be authentic in the HEV replication cycle, The pJE03-1760F/wt virus was inoculated into PLC/PRF/5 or A549 cells in six-well plates at 1.0×10^5 copies per well, and the cDNA-derived virus grew as efficiently as the original feces-derived virus in both PLC/PRF/5 and A549 cells, thus reaching 10^6 copies/ml on 30 dpi in the respective cells. Therefore, the reverse genetics system for HEV is utilizable in a robust cell-culture system and would be useful for the further elucidation of the

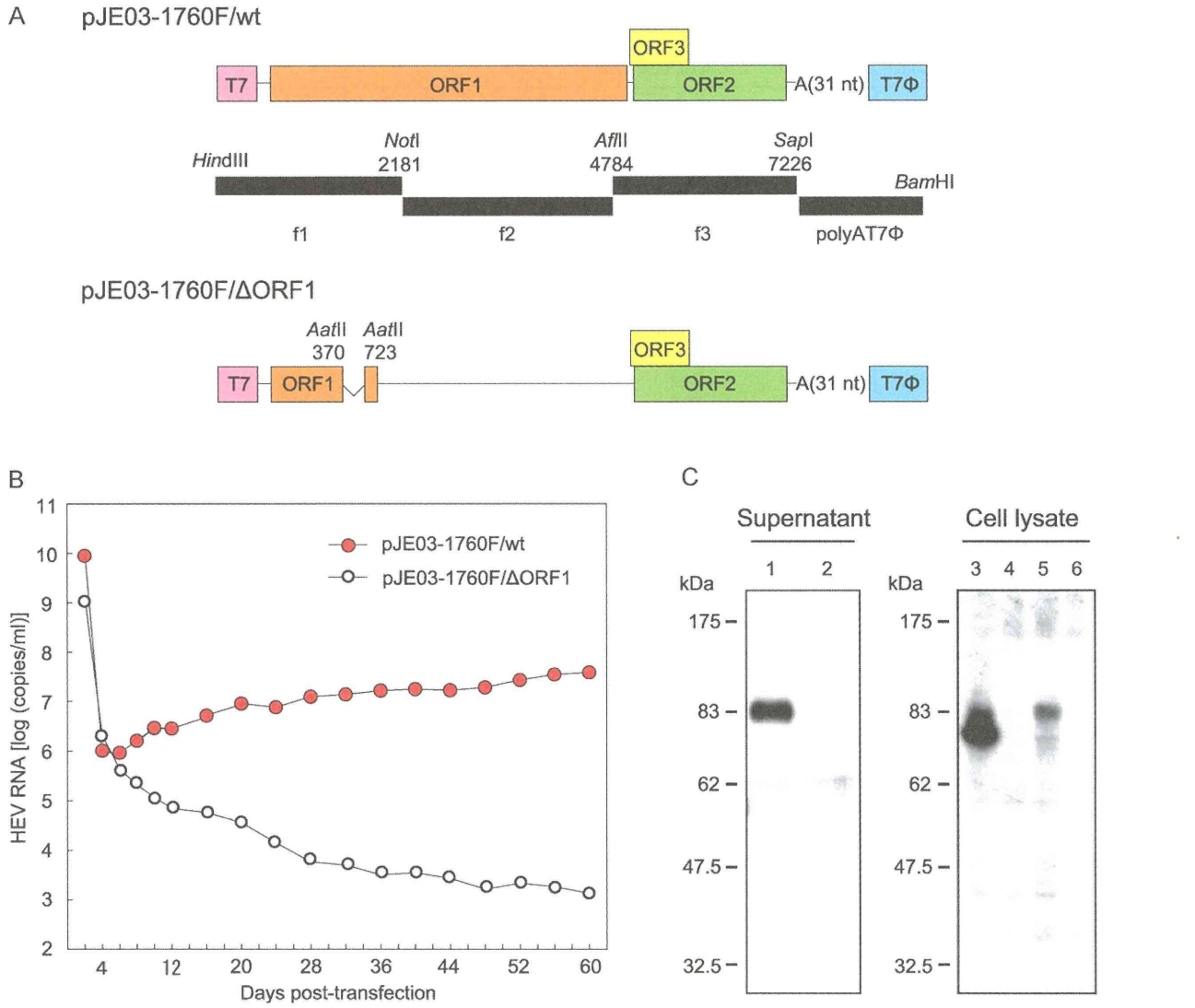


Fig. 4. Evaluation of a reverse genetics system for HEV using the JE03-1760F strain in cell culture. (A) Schematic diagram of full-length cDNA clones of the HEV JE03-1760F strain (pJE03-1760F/wt) and its ORF1-defective mutant (pJE03-1760F/ΔORF1). The pJE03-1760F/wt was constructed from four cDNA fragments (f1–f3 and polyAT7ϕ). The fragment polyAT7ϕ contains T7 terminator (ϕ) and 31-nt poly(A) sequences. (B) Quantification of HEV RNA in culture supernatants of RNA-transfected PLC/PRF/5 cells. The RNA titer in culture supernatants was measured by real-time RT-PCR: two wells were tested for each sample and the mean value was plotted. (C) ORF2 protein in culture supernatants or cell lysates of the RNA-transfected PLC/PRF/5 cells on 60 dpt was detected by Western blot with an anti-ORF2 mAb (H6210). The culture supernatant of cells transfected with RNA transcripts of pJE03-1760F/wt (lane 1) or pJE03-1760F/ΔORF1 (lane 2) as well as the lysate of cells transfected with RNA of pJE03-1760F/wt (lane 5) or pJE03-1760F/ΔORF1 (lane 6), were loaded. The lysate of cells transfected with pCI-HEVORF2 plasmid (lane 3) or mock (lane 4) was also loaded.
Modified from Yamada et al. (2009b).

mechanism of HEV replication and functional roles of HEV proteins.

5. Application of the HEV reverse genetics system to an analysis of the function of the ORF3 protein in virion egress

The ORF3 protein is phosphorylated and interacts not only with the ORF2 protein (Tyagi et al., 2002), but also with several cellular proteins including the cytoskeleton, α_1-microglobulin/bikunin precursor, tumor susceptibility gene 101 (Tsg101), and the src homology 3 domains (Korkaya et al., 2001; Surjit et al., 2006; Tyagi et al., 2004; Zafrullah et al., 1997). However, it is unclear whether the reported functions of ORF3 protein are authentic in the HEV life cycle and whether ORF3 protein is associated with virion morphogenesis and viral release. Previous studies using infectious HEV cDNA clones suggested that expression of the intact ORF3 protein is essential for infection of animals (Graff et al., 2005b; Huang et al., 2007), but is not required for infection and virion morphogenesis *in vitro* (Emerson et al., 2006b). However, an immuno-capture RT-PCR assay using an mAb against ORF3 protein (TA0536) suggests the presence of ORF3 protein on the surface of cell culture-generated HEV (Takahashi et al., 2008b). A full-length cDNA clone of an ORF3-deficient mutant (pJE03-1760F/ΔORF3; hereafter, ΔORF3 for simplicity) was constructed by mutating the third in-frame initiation codon of the ORF3 gene (ATG to GCA) on the wild-type pJE03-1760F/wt cDNA clone (Fig. 5A) to examine whether ORF3 protein is responsible for virion morphogenesis and viral release from infected cells (Yamada et al., 2009a), based on the report that the third in-frame initiation codon is necessary for ORF3 protein synthesis (Graff et al., 2006; Huang et al., 2007).

The transfection of PLC/PRF/5 cells with RNA transcripts of the ΔORF3 mutant gradually increased the HEV RNA level in the culture supernatant of the transfected cells from 16 dpt, reaching 5×10^6 copies/ml on 60 dpt, although the level was approximately 10-fold lower than that of the cells transfected with the wild-type RNA during the observation period of 16–60 dpt (Fig. 5B). A Western

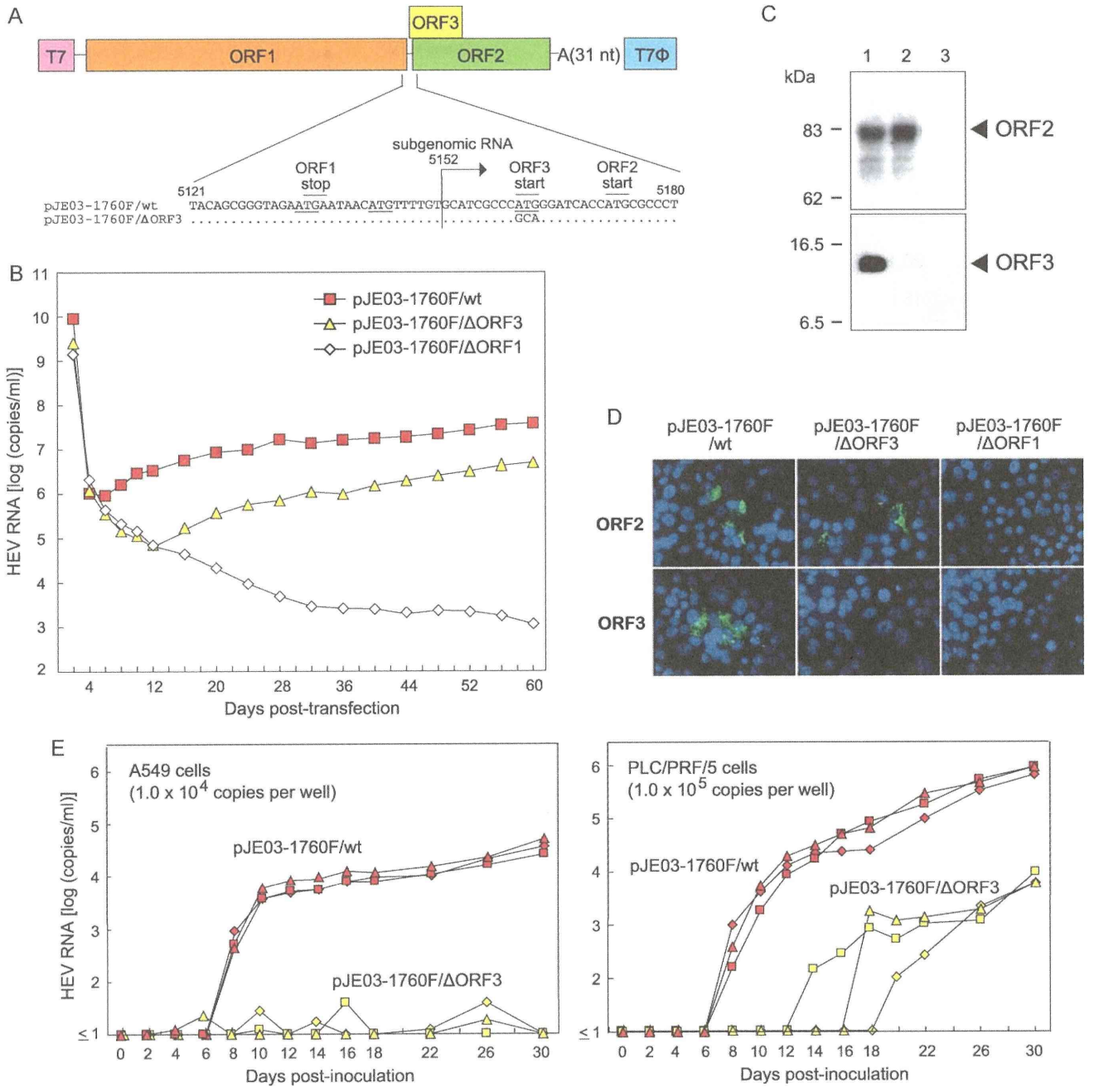


Fig. 5. Characterization of the ORF3-null mutant. (A) Schematic diagram of the full-length cDNA clone of the HEV JE03-1760F strain (pJE03-1760F/wt) and its derivative mutant. Nucleotide sequence of nt 5121–5180 of the full-length cDNA clones of pJE03-1760F/wt and pJE03-1760F/ΔORF3 are aligned. The first three in-frame ATG codons of the wild-type strain are underlined. The stop codon of the ORF1 gene and the proposed initiation codons of the ORF2 and ORF3 genes are indicated by overlines. Dots represent nucleotides identical to those at the top (wild-type). The initiation site of subgenomic mRNA transcription (Ichiyama et al., 2009) is depicted by a vertical bar with an arrow. The pJE03-1760F/ΔORF3 was generated by mutating ATG (Met) to GCA (Ala) at the third in-frame start codon of the ORF3 gene. (B) HEV RNA in culture supernatants of RNA-transfected PLC/PRF/5 cells was quantified by measuring the RNA titer in culture supernatants by real-time RT-PCR: two wells were tested for each sample and mean value was plotted. (C) On 60 dpt, viral proteins were detected by Western blotting with an anti-ORF2 mAb (upper panel) or anti-ORF3 mAb (lower panel): lane 1, pJE03-1760F/wt; lane 2, pJE03-1760F/ΔORF3; and lane 3, pJE03-1760F/ΔORF1. (D) Immunofluorescent staining of PLC/PRF/5 cells transfected with the transcribed RNAs from cDNA clones of the wild type (pJE03-1760F/wt) and its ORF3-null mutant (ΔORF3). The cells were incubated with an anti-ORF2 mAb (upper panel) or anti-ORF3 mAb (lower panel on 8 dpt) and then stained with Alexa Fluor 488-conjugated anti-mouse IgG. The nuclei were stained with DAPI. (E) Quantification of HEV RNA in culture supernatants of A549 or PLC/PRF/5 cells that were inoculated with the indicated viruses (in triplicate) and cultured for up to 30 days.
Modified from Yamada et al. (2009a).

blot analysis revealed expression of the ORF2 protein but not the ORF3 protein in the ΔORF3 mutant RNA-transfected cells on 60 dpt (Fig. 5C). As expected, ORF3 protein expression was not detectable by IFA in the PLC/PRF/5 cells transfected with RNA transcripts of ΔORF3 mutant, despite the clearly noticeable expression of ORF2 protein (Fig. 5D) (Yamada et al., 2009a). These results provide evidence that the third in-frame AUG codon (nt 5161–5163) is the authentic ORF3 start codon in the HEV replication cycle, consistent with the finding that the 2.2-kb subgenomic RNA of genotype 3 and 4 HEVs initiate at nt 5152 with the common sequence of 5′-GC (Ichiyama et al., 2009), which is identical to that of the prototype genotype 1 HEV (Graff et al., 2006) and is located between the second and third in-frame AUG codon in ORF3; the nucleotide positions are in accordance with the JE03-1760F strain. In addition,

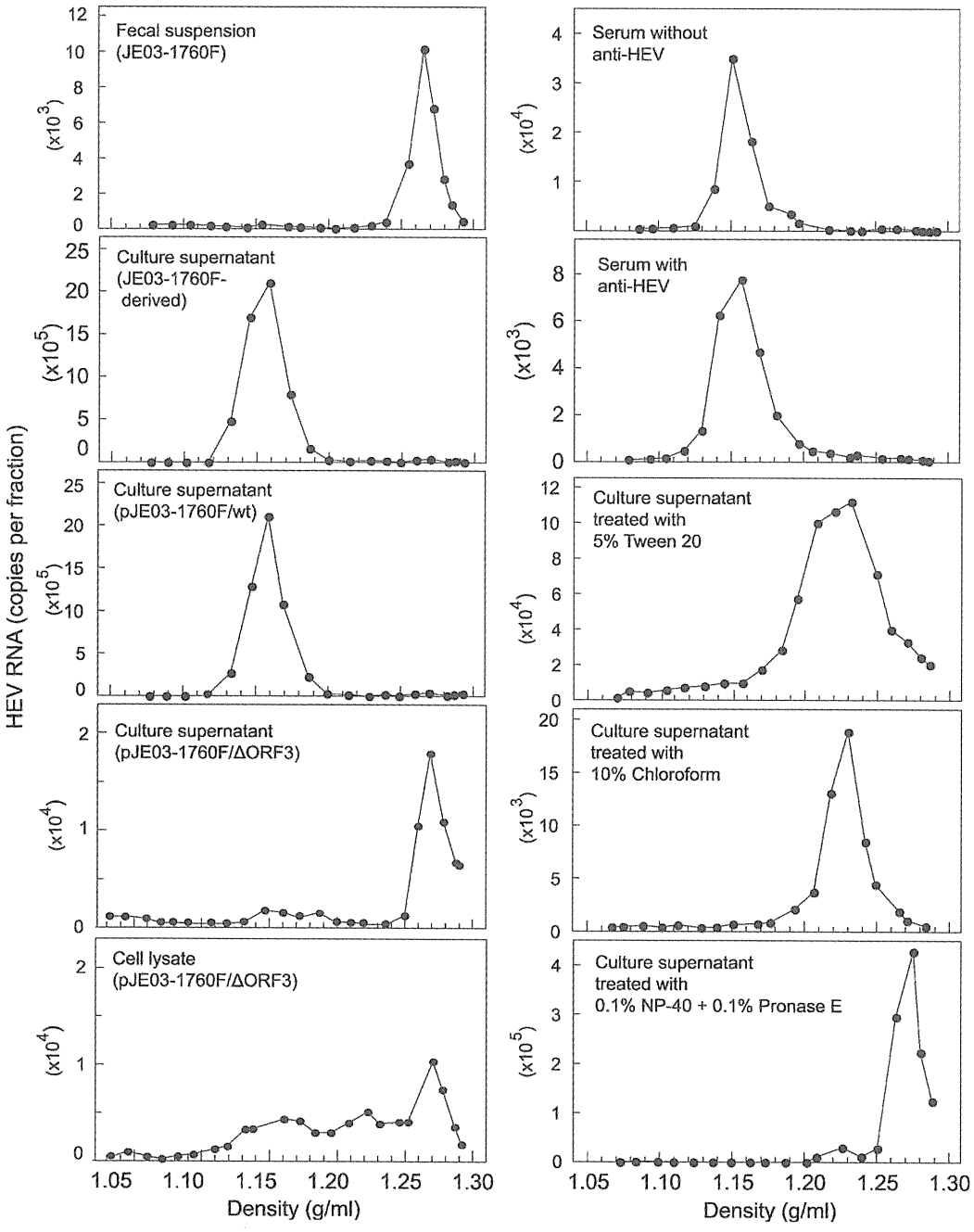


Fig. 6. Sucrose density-gradient fractionation of HEV in a fecal specimen (JE03-1760F), culture supernatants from PLC/PRF/5 cells inoculated with the JE03-1760F strain, or transfected with RNA transcripts of pJE03-1760F/wt, or pJE03-1760F/ΔORF3, lysates of PLC/PRF/5 cells transfected with RNA transcripts of pJE03-1760F/ΔORF3, serum samples with or without HEV antibodies, or JE03-1760F-derived culture supernatant treated with 5% Tween 20, 10% chloroform, or 0.1% NP-40 + 0.1 pronase E. Modified from Takahashi et al. (2010) and Yamada et al. (2009a).

the 2.2-kb subgenomic RNA of even the ΔORF3 mutant was found to initiate at nt 5152 (Fig. 5A) (Ichiyama et al., 2009).

The culture supernatant of the transfected cells was inoculated onto 3 wells each of PLC/PRF/5 or A549 cells at the HEV load of 10^5 or 10^4 copies per well, respectively, to determine whether the ΔORF3 mutant virus in the culture medium of the transfected cells is infectious and replication-competent and, if so, whether HEV virions are released from the ΔORF3 mutant-infected cultured cells (Fig. 5E). Viral RNA was nearly undetectable in the culture supernatant of A549 cells inoculated with the ΔORF3 mutant through the end of the observation period (30 dpi). PLC/PRF/5 cells inoculated with the ΔORF3 mutant showed less than 1/100 of the number of progenies in the culture medium in comparison to pJE03-1760F/wt: a sequence analysis excluded the possibility that the virus underwent a reversion to complement the deleterious mutations. Cell disruption was often observed during cell culture in the RNA- or mock-transfected PLC/PRF/5 cells and those inoculated with the culture supernatant of HEV-infected cells. The ΔORF3 mutant in the culture supernatant of the transfected cells bound to cells with an efficiency similar to that of the pJE03-1760F/wt virus in the cell lysate of the infected cells, in contrast to the wild-type virus in the culture supernatant with lower efficiency, and when applied to sucrose density ultracentrifugation, similar banding patterns peaking at 1.27–1.28 g/ml were observed in the culture supernatant and lysate of the ΔORF3 mutant RNA-transfected PLC/PRF/5 cells (Fig. 6). These results support the hypothesis that the ΔORF3

Table 3
Neutralization of HEV particles with either an anti-HEV immune serum or an mAbs (anti-ORF2 or anti-ORF3).

Inoculum (1.0×10^4 copies per well)	HEV replication in A549 cells						
	Without antibody[a]	Immune serum with anti-HEV IgG/IgM/IgA		Anti-ORF2 mAb (H6225)		Anti-ORF3 mAb (TA0536)	
		1:50	1:500	1 mg/ml	0.1 mg/ml	1 mg/ml	0.1 mg/ml
Fecal suspension (JE03-1760F)	+	–[b]	±[c]	–	±	+	+
Culture supernatant (JE03-1760F-derived)							
Nontreated	+	+	+	+	+	±	+
Treated with 5% Tween 20	+	±	±	±	±	±	±
Treated with 0.1% NP-40 + 0.1% pronase E	+	–	NT[d]	–	NT	+	NT
Serum with HEV RNA							
With anti-HEV	+	+	+	+	NT	+	NT
Without anti-HEV	+	+	+	+	NT	+	NT

Modified from Takahashi et al. (2008b, 2010)).
[a] Without prior incubation with anti-HEV antibody.
[b] Negative for HEV RNA throughout the observation period of 30 days after inoculation, thus representing complete neutralization.
[c] Inefficient replication with later appearance and lower level of HEV RNA in culture media, thus representing partial neutralization.
[d] NT, not tested.

mutant in the culture supernatant of the transfected or infected PLC/PRF/5 cells may be mostly an intracellular virus. Therefore, it is likely that the detectability of ΔORF3 mutant virus in the culture supernatant of the ΔORF3 mutant RNA-transfected PLC/PRF/5 cells and those inoculated with the mutant virus is due to the unauthentic release of intracellular virus by cell disruption. Despite the significantly lower levels of HEV RNA in the culture supernatant of ΔORF3 mutant-inoculated PLC/PRF/5 and A549 cells, intracellular HEV RNA was nearly equal to those in the respective cells inoculated with the pJE03-1760F/wt (Yamada et al., 2009a).

The immuno-capture RT-PCR assay indicated that ORF3 protein is present on the surface of at least 75% of HEV virions in the culture supernatant of pJE03-1760F/wt-transfected cells, despite the absence of ORF3 protein on fecal HEV (Table 2). The HEV particles in the culture supernatant of the pJE03-1760F/wt RNA-transfected cells banded at a density of 1.15–1.16 g/ml, which is markedly lower than that of HEV in a fecal suspension at 1.27–1.28 g/ml (Fig. 6). Therefore, HEV may be released from infected cells as a lipid-associated virion, accompanied by ORF3 protein. This hypothesis is consistent with the observation that the ORF3 protein binds to the ORF2 capsid protein (Tyagi et al., 2002) and to cellular Tsg101 (Surjit et al., 2006). Tsg101 binds to the PSAP motif located within the ORF3 protein, and the PSAP motif is conserved in all HEV isolates, including avian HEV. Tsg101 has been identified as the critical cellular protein required for budding of enveloped viruses, i.e., human immunodeficiency virus type-1 (HIV) and Ebola virus, from the plasma membrane (Garrus et al., 2001; Martin-Serrano et al., 2001). Tsg101 is recruited to the sites of virus budding by binding to the P(S/T)AP motif located within the HIV Gag and the Ebola VP40 matrix proteins. The release of genotype 1 HEV from cultured Huh-7 hepatoma cells depends on ORF3 protein and requires an intact PXXP motif (Emerson et al., 2010). Taken together, it is likely that the ORF3 protein promotes budding of "enveloped" HEV particles by recruiting Tsg101.

The application of the reverse genetics system for HEV utilizable in a robust cell culture system revealed that virion egress from infected cells is dependent on ORF3 protein. In addition, these studies indicated that the ORF3 protein is present on the surface of HEV particles released from infected cells, and suggested that the HEV particles released from infected cells are thus lipid-associated, which has recently been confirmed by Emerson et al. (2010).

6. Propagation of HEV strains in circulating blood in cultured cells

Infection of humans with HEV via blood transfusion was reported not only in developing countries (genotype 1) (Arankalle and Chobe, 2000), but also in industrialized countries, including Japan (genotypes 3 and 4) (Matsubayashi et al., 2008; Mitsui et al., 2004), thus suggesting that HEV in serum samples can also be propagated in cultured cells. Therefore, further experiments examined whether various HEV strains in serum samples obtained from 23 patients with genotype 1, 3, or 4 HEV can replicate in PLC/PRF/5 and A549 cells and release infectious progenies into culture media, in relation to the HEV load, genotype, and co-existence of HEV antibodies (Takahashi et al., 2010).

Various HEV strains of genotype 1, 3, or 4 in serum samples obtained from patients with domestic or imported hepatitis E can also infect and replicate efficiently in PLC/PRF/5 and A549 cells. Of note, HEV strains in all serum samples tested were successfully propagated in cultured cells when inoculated at an HEV load of $\geq 3.5 \times 10^4$ copies per well in a six-well microplate, with the HEV load reaching 4.7×10^3–7.2×10^6 copies/ml on 30 dpi, while HEV strains in only 30% of serum samples grew in cultured cells upon inoculation at 2.0×10^4 copies per well. In other words, PLC/PRF/5 and A549 cells can support multiplication of HEV strains in all serum samples with or without concurrent HEV antibodies, irrespective of the HEV genotype if the HEV load in serum is 3.5×10^5 copies/ml or higher. Progenies of serum-derived HEV strains in culture supernatant were successfully passaged in PLC/PRF/5 and A549 cells, indicating that HEV progenies of serum origin released from cultured cells are infectious, similar to those of feces origin (Takahashi et al., 2010).

7. Characterization of HEV particles in circulating blood, in comparison to those in culture supernatant and feces

HEV particles in the serum samples were characterized and compared with those in the culture supernatant and feces to clarify the reason why HEV strains in sera are infectious in cultured cells despite the presence of HEV antibodies. Over 90% of HEV particles in the circulation were free of immunoglobulins even in the presence of IgM HEV antibodies. Similar to cell culture-generated HEV particles, HEV particles in serum were non-neutralizable by immune sera and anti-ORF2 mAb that can definitely neutralize

the infection of HEV in feces in the cell culture system (Table 3) (Takahashi et al., 2008a; Tanaka et al., 2007), and banded at a sucrose density of 1.15–1.16 g/ml, irrespective of the presence or absence of circulating anti-HEV antibodies (Fig. 6), which was identical to that in culture supernatant but markedly lower than that in feces at 1.27–1.28 g/ml. Few or no virus particles in both serum and cell culture were captured by anti-ORF2 mAb and anti-ORF3 mAb without prior treatment with detergent (Table 2). However, treatment with 5% Tween 20 markedly increased the binding efficiency of HEV particles in serum by anti-ORF2 mAb and anti-ORF3 mAb to 55.7% and 90.4%, respectively, showing a similar pattern as cell culture-produced HEV particles (Table 2) (Takahashi et al., 2010). These results suggest that the HEV virion in serum possesses the ORF3 protein on its surface in association with lipids, similar to those in the supernatant of cultured cells (Yamada et al., 2009a), and would reconcile the apparently contradictory observations that IgM and IgA antibodies, capable of viral neutralization, and infectious virus co-exist in acute-phase serum specimens, as reported for HAV (Lemon and Binn, 1985; Purcell et al., 1984).

The partial neutralization of the detergent-treated HEV virions in cell culture by anti-HEV antibodies (Table 3) may be ascribable to incomplete exposure of ORF2 and ORF3 proteins after treatment with detergent only. This is consistent with the observation that 10–44% of the 5% Tween 20-treated HEV particles in cell culture and serum were not captured by anti-ORF2 and anti-ORF3 mAbs in the immuno-capture studies (Table 2). The cell culture-produced HEV particles treated with both detergent and protease, with a buoyant density of 1.27–1.28 g/ml in sucrose, can be neutralized by an anti-HEV immune serum and anti-ORF2 mAb, indicating that virions treated with lipid solvent and protease, possessing the same characteristics as virions from feces (see Table 2 and Fig. 6), are neutralizable by anti-HEV immune sera. The ORF3 protein is likely to act as an adapter to link various intracellular transduction pathways, creating the host cell environment favorable for HEV replication and assembly (Chandra et al., 2008). As mentioned above (see Section 5), the ORF3 protein is required for virion release from cultured cells. Taken together, it is very likely that HEV particles are released from both infected cultured cells (*in vitro*) and infected hepatocytes (*in vivo*) as lipid-associated virions, accompanied by ORF3 protein, and that the ORF3 protein and lipids are dissociated from the virion after shedding in bile duct and then duodenum which contain detergent (deoxycholic acid) and protease (trypsin), respectively.

No putative receptor for HEV has been isolated, and virtually nothing is known about the mechanism by which HEV enters susceptible cells. A previous study on virus attachment determined that cloned pJE03-1760F/wt virus in culture medium adsorbed to A549 cells with lower efficiency than that in cell lysate, peaking at the sucrose density of 1.27–1.28 g/ml (approximately 3% and 14%, respectively, of the input virus within 1 h) (Yamada et al., 2009a), thus suggesting that, although inefficiently, non-neutralizable HEV particles in serum samples and culture supernatant, which are associated with lipids and band at 1.15–1.16 g/ml in sucrose gradients (Fig. 6), can bind to cultured cells. As indicated in Table 3, even non-neutralizable HEV particles in serum samples and culture supernatant can be propagated in cultured cells. Of interest, a lipid solvent did not abolish or increase the infectivity of HEV progenies from infected cell culture, and both cell culture-generated HEV particles and those that were treated with detergent and/or protease were propagated in cultured cells with nearly identical efficiency (Takahashi et al., 2010), regardless of the presence or the absence of the ORF3 protein and lipids on the surface. Taken altogether, it seems unlikely that only a fraction of HEV particles in serum samples and culture supernatant, partially unaccompanied by lipids, can bind to the cell surface receptor and enter susceptible cells. Future studies must determine whether and how non-neutralizable HEV particles in serum and culture supernatant can enter susceptible cells in order to provide a plausible explanation(s) for the unexpected and intriguing observed phenomenon.

8. Conclusion

The lack of an efficient cell culture system for HEV has greatly hampered the performance of detailed analyses of the viral replication cycle in infected cells to resolve many important questions. This laboratory developed the first efficient cell culture systems for HEV, capable of releasing infectious HEV progenies in high titers into culture media, using PLC/PRF/5 cells originating from human hepatocellular carcinoma and A549 cells from human lung cancer as host cells. The success with the original genotype 3 JE03-1760F strain has been extended to the genotype 4 HE-JF5/15F strain that can bring about the replication of HEV with an even higher efficiency and that can be passaged through many generations, and to various HEV strains in serum samples obtained from patients with genotype 1, 3, or 4 HEV. A key to successful propagation in culture would be a high infectious capacity of the primary inoculum, i.e., the concentration of virus in copies/ml. In addition, infectious HEV cDNA clones of the wild-type JE03-1760F strain and its variants have been engineered. These cell culture systems for HEV, reinforced by reverse genetics, will solve many mysteries and answer numerous questions surrounding the epidemiology, virus itself, and life cycle of HEV including viral absorption/entry, packaging and release.

Cell culture-generated HEV particles and those in circulating blood are found to be associated with lipids and ORF3 protein, thereby likely contributing to the assembly and release of HEV from non-cytopathically infected cells *in vivo* and *in vitro*. Although lipid-associated virions of other non-enveloped viruses such as HAV have been reported (Lemon and Binn, 1985; Provost et al., 1975; Rueckert, 1976), the underlying mechanism by which HEV particles in blood and culture supernatant can be propagated in cultured cells, despite the fact that they are seemingly "enveloped" and non-neutralizable by immune sera, still needs to be clarified in future studies.

References

Agrawal, S., Gupta, D., Panda, S.K., 2001. The 3′ end of hepatitis E virus (HEV) genome binds specifically to the viral RNA-dependent RNA polymerase (RdRp). Virology 282 (1), 87–101.

Arankalle, V.A., Chobe, L.P., 2000. Retrospective analysis of blood transfusion recipients: evidence for post-transfusion hepatitis E. Vox Sang. 79 (2), 72–74.

Arankalle, V.A., Ticehurst, J., Sreenivasan, M.A., Kapikian, A.Z., Popper, H., Pavri, K.M., Purcell, R.H., 1988. Aetiological association of a virus-like particle with enterically transmitted non-A, non-B hepatitis. Lancet 1 (8585), 550–554.

Balayan, M.S., Andjaparidze, A.G., Savinskaya, S.S., Ketiladze, E.S., Braginsky, D.M., Savinov, A.P., Poleschuk, V.F., 1983. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology 20 (1), 23–31.

Chandra, V., Taneja, S., Kalia, M., Jameel, S., 2008. Molecular biology and pathogenesis of hepatitis E virus. J. Biosci. 33 (4), 451–464.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D., Gerolami, R., 2010. Pig liver sausage as a source of hepatitis E virus transmission to humans. J. Infect. Dis. 202 (6), 825–834.

Dalton, H.R., Bendall, R., Ijaz, S., Banks, M., 2008. Hepatitis E: an emerging infection in developed countries. Lancet Infect. Dis. 8 (11), 698–709.

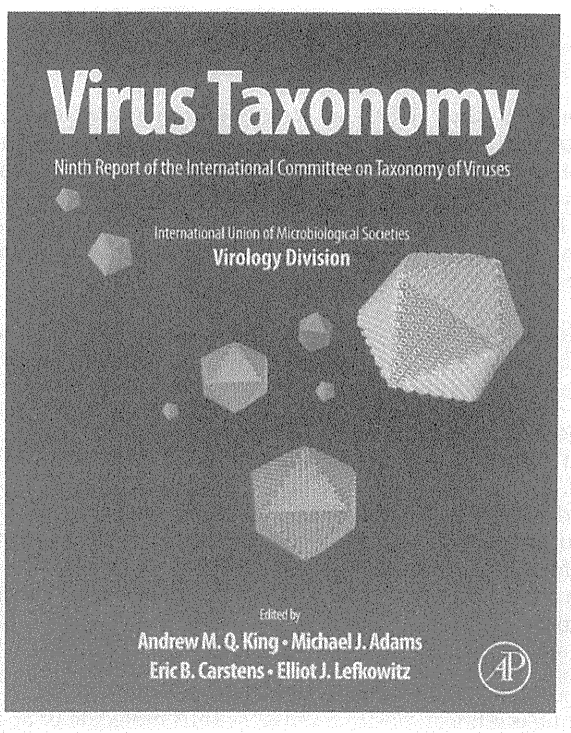
Emerson, S.U., Anderson, D., Arankalle, A., Meng, X.J., Purdy, M., Schlauder, G.G., Tsarev, S.A., 2005a. Hepevirus. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy, VIIIth Report of the ICTV. Elsevier/Academic Press, London, pp. 853–857.

Emerson, S.U., Arankalle, V.A., Purcell, R.H., 2005b. Thermal stability of hepatitis E virus. J. Infect. Dis. 192 (5), 930–933.

Emerson, S.U., Clemente-Casares, P., Moiduddin, N., Arankalle, V.A., Torian, U., Purcell, R.H., 2006a. Putative neutralization epitopes and broad cross-genotype neutralization of Hepatitis E virus confirmed by a quantitative cell-culture assay. J. Gen. Virol. 87 (Pt 3), 697–704.

Emerson, S.U., Nguyen, H., Torian, U., Purcell, R.H., 2006b. ORF3 protein of hepatitis E virus is not required for replication, virion assembly, or infection of hepatoma cells in vitro. J. Virol. 80 (21), 10457–10464.
Emerson, S.U., Nguyen, H.T., Torian, U., Burke, D., Engle, R., Purcell, R.H., 2010. Release of genotype 1 hepatitis E virus from cultured hepatoma and polarized intestinal cells depends on open reading frame 3 protein and requires an intact PXXP motif. J. Virol. 84 (18), 9059–9069.
Emerson, S.U., Purcell, R.H., 2007. Hepatitis E virus. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology. , fifth ed. Lippincott Williams & Wilkins, Philadelphia, pp. 3047–3058.
Garrus, J.E., von Schwedler, U.K., Pornillos, O.W., Morham, S.G., Zavitz, K.H., Wang, H.E., Wettstein, D.A., Stray, K.M., Cote, M., Rich, R.L., Myszka, D.G., Sundquist, W.I., 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell 107 (1), 55–65.
Graff, J., Kasang, C., Normann, A., Pfisterer-Hunt, M., Feinstone, S.M., Flehmig, B., 1994. Mutational events in consecutive passages of hepatitis A virus strain GBM during cell culture adaptation. Virology 204 (1), 60–68.
Graff, J., Nguyen, H., Kasorndorkbua, C., Halbur, P.G., St Claire, M., Purcell, R.H., Emerson, S.U., 2005a. In vitro and in vivo mutational analysis of the 3′-terminal regions of hepatitis e virus genomes and replicons. J. Virol. 79 (2), 1017–1026.
Graff, J., Nguyen, H., Yu, C., Elkins, W.R., St Claire, M., Purcell, R.H., Emerson, S.U., 2005b. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. J. Virol. 79 (11), 6680–6689.
Graff, J., Torian, U., Nguyen, H., Emerson, S.U., 2006. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. J. Virol. 80 (12), 5919–5926.
Graff, J., Zhou, Y.H., Torian, U., Nguyen, H., St Claire, M., Yu, C., Purcell, R.H., Emerson, S.U., 2008. Mutations within potential glycosylation sites in the capsid protein of hepatitis E virus prevent the formation of infectious virus particles. J. Virol. 82 (3), 1185–1194.
Guu, T.S., Liu, Z., Ye, Q., Mata, D.A., Li, K., Yin, C., Zhang, J., Tao, Y.J., 2009. Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. Proc. Natl. Acad. Sci. U.S.A. 106 (31), 12992–12997.
Hu, N.Z., Hu, Y.Z., Shi, H.J., Liu, G.D., Qu, S., 2002. Mutational characteristics in consecutive passage of rapidly replicating variants of hepatitis A virus strain H2 during cell culture adaptation. World J. Gastroenterol. 8 (5), 872–878.
Huang, R., Li, D., Wei, S., Li, Q., Yuan, X., Geng, L., Li, X., Liu, M., 1999. Cell culture of sporadic hepatitis E virus in China. Clin. Diagn. Lab. Immunol. 6 (5), 729–733.
Huang, R., Nakazono, N., Ishii, K., Li, D., Kawamata, O., Kawaguchi, R., Tsukada, Y., 1995. Hepatitis E virus (87A strain) propagated in A549 cells. J. Med. Virol. 47 (4), 299–302.
Huang, R.T., Li, D.R., Wei, J., Huang, X.R., Yuan, X.T., Tian, X., 1992. Isolation and identification of hepatitis E virus in Xinjiang, China. J. Gen. Virol. 73 (Pt 5), 1143–1148.
Huang, Y.W., Opriessnig, T., Halbur, P.G., Meng, X.J., 2007. Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. J. Virol. 81 (6), 3018–3026.
Ichiyama, K., Yamada, K., Tanaka, T., Nagashima, S., Jirintai, Takahashi, M., Okamoto, H., 2009. Determination of the 5′-terminal sequence of subgenomic RNA of hepatitis E virus strains in cultured cells. Arch. Virol. 154 (12), 1945–1951.
Jansen, R.W., Newbold, J.E., Lemon, S.M., 1988. Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for in vitro replication. Virology 163 (2), 299–307.
Kabrane-Lazizi, Y., Meng, X.J., Purcell, R.H., Emerson, S.U., 1999. Evidence that the genomic RNA of hepatitis E virus is capped. J. Virol. 73 (10), 8848–8850.
Kane, M.A., Bradley, D.W., Shrestha, S.M., Maynard, J.E., Cook, E.H., Mishra, R.P., Joshi, D.D., 1984. Epidemic non-A, non-B hepatitis in Nepal. Recovery of a possible etiologic agent and transmission studies in marmosets. JAMA 252 (22), 3140–3145.
Kazachkov Yu, A., Balayan, M.S., Ivannikova, T.A., Panina, L.I., Orlova, T.M., Zamyatina, N.A., Kusov, Y., 1992. Hepatitis E virus in cultivated cells. Arch. Virol. 127 (1–4), 399–402.
Koonin, E.V., Gorbalenya, A.E., Purdy, M.A., Rozanov, M.N., Reyes, G.R., Bradley, D.W., 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. Proc. Natl. Acad. Sci. U.S.A. 89 (17), 8259–8263.
Korkaya, H., Jameel, S., Gupta, D., Tyagi, S., Kumar, R., Zafrullah, M., Mazumdar, M., Lal, S.K., Xiaofang, L., Sehgal, D., Das, S.R., Sahal, D., 2001. The ORF3 protein of hepatitis E virus binds to Src homology 3 domains and activates MAPK. J. Biol. Chem. 276 (45), 42389–42400.
Lemon, S.M., Binn, L.N., 1985. Incomplete neutralization of hepatitis A virus in vitro due to lipid-associated virions. J. Gen. Virol. 66 (Pt 11), 2501–2505.
Lewis, H.C., Wichmann, O., Duizer, E., 2010. Transmission routes and risk factors for autochthonous hepatitis E virus infection in Europe: a systematic review. Epidemiol. Infect. 138 (2), 145–166.
Li, D.R., Huang, R.T., Pang, J.J., Yuan, X.T., Li, X.Y., 1996. Biological feature and genome analysis of HEV isolated by cell-culture. In: Buisson, Y., Coursaget, P., Kane, M. (Eds.), Enterically-Transmitted Hepatitis Viruses. La Simarre, Joue-les-Tours, France, pp. 349–361.
Lorenzo, F.R., Tanaka, T., Takahashi, H., Ichiyama, K., Hoshino, Y., Yamada, K., Inoue, J., Takahashi, M., Okamoto, H., 2008. Mutational events during the primary propagation and consecutive passages of hepatitis E virus strain JE03-1760F in cell culture. Virus Res. 137 (1), 86–96.
Lu, L., Li, C., Hagedorn, C.H., 2006. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Rev. Med. Virol. 16 (1), 5–36.
Martin-Serrano, J., Zang, T., Bieniasz, P.D., 2001. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. Nat. Med. 7 (12), 1313–1319.
Matsubayashi, K., Kang, J.H., Sakata, H., Takahashi, K., Shindo, M., Kato, M., Sato, S., Kato, T., Nishimori, H., Tsuji, K., Maguchi, H., Yoshida, J., Maekubo, H., Mishiro, S., Ikeda, H., 2008. A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route. Transfusion 48 (7), 1368–1375.
Meng, J., Dubreuil, P., Pillot, J., 1997a. A new PCR-based seroneutralization assay in cell culture for diagnosis of hepatitis E. J. Clin. Microbiol. 35 (6), 1373–1377.
Meng, J., Guinet, R., Pillot, J., 1996. Infection of PLC/PRF-5 cells with the hepatitis E virus. In: Buisson, Y., Coursaget, P., Kane, M. (Eds.), Enterically-transmitted hepatitis viruses. La Simarre, Joue-les-Tours, France, pp. 336–345.
Meng, X.J., 2010. Hepatitis E virus: animal reservoirs and zoonotic risk. Vet. Microbiol. 140 (3–4), 256–265.
Meng, X.J., Purcell, R.H., Halbur, P.G., Lehman, J.R., Webb, D.M., Tsareva, T.S., Haynes, J.S., Thacker, B.J., Emerson, S.U., 1997b. A novel virus in swine is closely related to the human hepatitis E virus. Proc. Natl. Acad. Sci. U.S.A. 94 (18), 9860–9865.
Mitsui, T., Tsukamoto, Y., Yamazaki, C., Masuko, K., Tsuda, F., Takahashi, M., Nishizawa, T., Okamoto, H., 2004. Prevalence of hepatitis E virus infection among hemodialysis patients in Japan: evidence for infection with a genotype 3 HEV by blood transfusion. J. Med. Virol. 74 (4), 563–572.
Okamoto, H., 2007. Genetic variability and evolution of hepatitis E virus. Virus Res. 127 (2), 216–228.
Okamoto, H., Takahashi, M., Nishizawa, T., 2003. Features of hepatitis E virus infection in Japan. Intern. Med. 42 (11), 1065–1071.
Pavio, N., Meng, X.J., Renou, C., 2010. Zoonotic hepatitis E: animal reservoirs and emerging risks. Vet. Res. 41 (6), 46.
Pillot, J., Sharma, M.D., Lazizi, Y., Budkowska, A., Dauguet, C., Galimand, M., Sarthou, J.L., 1987. Immunological characterization of a viral agent involved in epidemic and sporadic non A non B hepatitis. Am. Inst. Pasteur/Virologie 138, 145–158.
Provost, P.J., Wolanski, B.S., Miller, W.J., Ittensohn, O.L., McAleer, W.J., Hilleman, M.R., 1975. Physical, chemical and morphologic dimensions of human hepatitis A virus strain CR326 (38578). Proc. Soc. Exp. Biol. Med. 148 (2), 532–539.
Purcell, R.H., Emerson, S.U., 2001. Animal models of hepatitis A and E. ILAR J. 42 (2), 161–177.
Purcell, R.H., Emerson, S.U., 2008. Hepatitis E: an emerging awareness of an old disease. J. Hepatol. 48 (3), 494–503.
Purcell, R.H., Feinstone, S.M., Ticehurst, J.R., Daemer, R.J., Baroudy, B.M., 1984. Hepatitis A virus. In: Vyas, G.N., Dienstag, J.L., Hoofnagle, J.H. (Eds.), Viral Hepatitis and Liver Diseases. Grune & Stratton, Orland, FL, pp. 9–22.
Reuter, G., Fodor, D., Forgach, P., Katai, A., Szucs, G., 2009. Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. J. Clin. Virol. 44 (4), 277–281.
Reyes, G.R., Purdy, M.A., Kim, J.P., Luk, K.C., Young, L.M., Fry, K.E., Bradley, D.W., 1990. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 247 (4948), 1335–1339.
Rueckert, R., 1976. On the structure and morphogenesis of picornaviruses. In: Frankel-Conrat, H., Wagner, R.R. (Eds.), Comprehensive Virology, vol. 6. Plenum Press, New Yor, NY, pp. 131–213.
Sonoda, H., Abe, M., Sugimoto, T., Sato, Y., Bando, M., Fukui, E., Mizuo, H., Takahashi, M., Nishizawa, T., Okamoto, H., 2004. Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. J. Clin. Microbiol. 42 (11), 5371–5374.
Surjit, M., Jameel, S., Lal, S.K., 2007. Cytoplasmic localization of the ORF2 protein of hepatitis E virus is dependent on its ability to undergo retrotranslocation from the endoplasmic reticulum. J. Virol. 81 (7), 3339–3345.
Surjit, M., Oberoi, R., Kumar, R., Lal, S.K., 2006. Enhanced alpha1 microglobulin secretion from Hepatitis E virus ORF3-expressing human hepatoma cells is mediated by the tumor susceptibility gene 101. J. Biol. Chem. 281 (12), 8135–8142.
Takahashi, K., Kitajima, N., Abe, N., Mishiro, S., 2004a. Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. Virology 330 (2), 501–505.
Takahashi, K., Toyota, J., Karino, Y., Kang, J.H., Maekubo, H., Abe, N., Mishiro, S., 2004b. Estimation of the mutation rate of hepatitis E virus based on a set of closely related 7.5-year-apart isolates from Sapporo, Japan. Hepatol. Res. 29 (4), 212–215.
Takahashi, M., Hoshino, Y., Tanaka, T., Takahashi, H., Nishizawa, T., Okamoto, H., 2008a. Production of monoclonal antibodies against hepatitis E virus capsid protein and evaluation of their neutralizing activity in a cell culture system. Arch. Virol. 153 (4), 657–666.
Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F., Okamoto, H., 2003. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J. Gen. Virol. 84 (Pt 4), 851–862.
Takahashi, M., Tanaka, T., Azuma, M., Kusano, E., Aikawa, T., Shibayama, T., Yazaki, Y., Mizuo, H., Inoue, J., Okamoto, H., 2007. Prolonged fecal shedding of hepatitis E virus (HEV) during sporadic acute hepatitis E: evaluation of infectivity of HEV in fecal specimens in a cell culture system. J. Clin. Microbiol. 45 (11), 3671–3679.
Takahashi, M., Tanaka, T., Takahashi, H., Hoshino, Y., Nagashima, S., Jirintai, Mizuo, H., Yazaki, Y., Takagi, T., Azuma, M., Kusano, E., Isoda, N., Sugano, K., Okamoto, H., 2010. Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: char-

acterization of HEV virions in blood circulation. J. Clin. Microbiol. 48 (4), 1112–1125.
Takahashi, M., Yamada, K., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T., Okamoto, H., 2008b. Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces. Arch. Virol. 153 (9), 1703–1713.
Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.C., Bradley, D.W., Fry, K.E., Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185 (1), 120–131.
Tam, A.W., White, R., Reed, E., Short, M., Zhang, Y., Fuerst, T.R., Lanford, R.E., 1996. In vitro propagation and production of hepatitis E virus from in vivo-infected primary macaque hepatocytes. Virology 215 (1), 1–9.
Tam, A.W., White, R., Yarbough, P.O., Murphy, B.J., McAtee, C.P., Lanford, R.E., Fuerst, T.R., 1997. In vitro infection and replication of hepatitis E virus in primary cynomolgus macaque hepatocytes. Virology 238 (1), 94–102.
Tanaka, T., Takahashi, M., Kusano, E., Okamoto, H., 2007. Development and evaluation of an efficient cell-culture system for Hepatitis E virus. J. Gen. Virol. 88 (Pt 3), 903–911.
Tanaka, T., Takahashi, M., Takahashi, H., Ichiyama, K., Hoshino, Y., Nagashima, S., Mizuo, H., Okamoto, H., 2009. Development and characterization of a genotype 4 hepatitis E virus cell culture system using a HE-JF5/15F strain recovered from a fulminant hepatitis patient. J. Clin. Microbiol. 47 (6), 1906–1910.
Tei, S., Kitajima, N., Takahashi, K., Mishiro, S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362 (9381), 371–373.
Torresi, J., Li, F., Locarnini, S.A., Anderson, D.A., 1999. Only the non-glycosylated fraction of hepatitis E virus capsid (open reading frame 2) protein is stable in mammalian cells. J. Gen. Virol. 80 (Pt 5), 1185–1188.
Tyagi, S., Korkaya, H., Zafrullah, M., Jameel, S., Lal, S.K., 2002. The phosphorylated form of the ORF3 protein of hepatitis E virus interacts with its non-glycosylated form of the major capsid protein, ORF2. J. Biol. Chem. 277 (25), 22759–22767.
Tyagi, S., Surjit, M., Roy, A.K., Jameel, S., Lal, S.K., 2004. The ORF3 protein of hepatitis E virus interacts with liver-specific alpha1-microglobulin and its precursor alpha1-microglobulin/bikunin precursor (AMBP) and expedites their export from the hepatocyte. J. Biol. Chem. 279 (28), 29308–29319.
Wei, S., Walsh, P., Huang, R., To, S.S., 2000. 93G, a novel sporadic strain of hepatitis E virus in South China isolated by cell culture. J. Med. Virol. 61 (3), 311–318.
Williams, T.P., Kasorndorkbua, C., Halbur, P.G., Haqshenas, G., Guenette, D.K., Toth, T.E., Meng, X.J., 2001. Evidence of extrahepatic sites of replication of the hepatitis E virus in a swine model. J. Clin. Microbiol. 39 (9), 3040–3046.
Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Nagashima, S., Tanaka, T., Okamoto, H., 2009a. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. J. Gen. Virol. 90 (Pt 8), 1880–1891.
Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Tanaka, T., Okamoto, H., 2009b. Construction of an infectious cDNA clone of hepatitis E virus strain JE03-1760F that can propagate efficiently in cultured cells. J. Gen. Virol. 90 (Pt 2), 457–462.
Yamashita, T., Mori, Y., Miyazaki, N., Cheng, R.H., Yoshimura, M., Unno, H., Shima, R., Moriishi, K., Tsukihara, T., Li, T.C., Takeda, N., Miyamura, T., Matsuura, Y., 2009. Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. Proc. Natl. Acad. Sci. U.S.A. 106 (31), 12986–12991.
Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y., Okamoto, H., 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J. Gen. Virol. 84 (Pt 9), 2351–2357.
Zafrullah, M., Ozdener, M.H., Panda, S.K., Jameel, S., 1997. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J. Virol. 71 (12), 9045–9053.



From David A. Anderson, Hiroaki Okamoto, Suzanne U. Emerson, Shahid Jameel, Tim J. Harrison, Vidya A. Arankalle, and Xiang-Jin Meng, Hepeviridae. In: Andrew M.Q. King, Michael J. Adams, Eric B. Carstens, and Elliot J. Lefkowitz, editors, Virus Taxonomy. Oxford: Elsevier, 2011, pp. 1021-1028.
ISBN: 978-0-12-384684-6

FAMILY *HEPEVIRIDAE*

Taxonomic structure of the family

Family	*Hepeviridae*
Genus	*Hepevirus*

Virion properties

MORPHOLOGY

Virions of hepatitis E virus (HEV) are icosahedral, non-enveloped, spherical particles with a diameter of approximately 27–34 nm (Figure 1). The capsid is formed by capsomeres consisting of homodimers of a single capsid protein forming the virus shell. Each capsid protein contains three linear domains forming distinct structural elements: S (the continuous capsid), P1 (three-fold protrusions), and P2 (two-fold spikes). Each domain contains a putative polysaccharide-binding site that may interact with cellular receptors. Native T = 3 capsid contains flat dimers, with less curvature than those of T = 1 virus-like particles (Figure 2).

Virion particles of avian hepatitis E virus (Figure 3) revealed by negative staining EM of bile samples from chickens with hepatitis–splenomegaly syndrome are similar in size and morphology to members of the genus *Hepevirus*.

PHYSICOCHEMICAL AND PHYSICAL PROPERTIES

Virion buoyant density is 1.35 to 1.40 g cm^{-3} in CsCl and 1.29 g cm^{-3} in glycerol and potassium tartrate gradients. Virion $S_{20,w}$ is 183S. Virion is sensitive to low-temperature storage (between −70 °C and +8 °C) and iodinated disinfectants. The virion of hepatitis E virus is more heat-labile than is hepatitis A virus: hepatitis E virus was about 50% inactivated at 56 °C and almost totally inactivated (96%) at 60 °C for 1 h. Liver suspensions containing avian hepatitis E virus remained infectious after treatment with chloroform and ether but lost infectivity after incubating at 56 °C for 1 h or 37 °C for 6 h. Viral infectivity in liver suspensions was reduced 1000-fold after treatment with 0.05% Tween-20, 0.1% NP40 and 0.05% formalin.

NUCLEIC ACID

The HEV genome is a linear, positive sense, ssRNA molecule of approximately 7.2 kb, with a 5′-m^{7}G cap structure and a 3′-poly(A) tail. The genome of avian hepatitis E virus is similar but only about 6.6 kb in size.

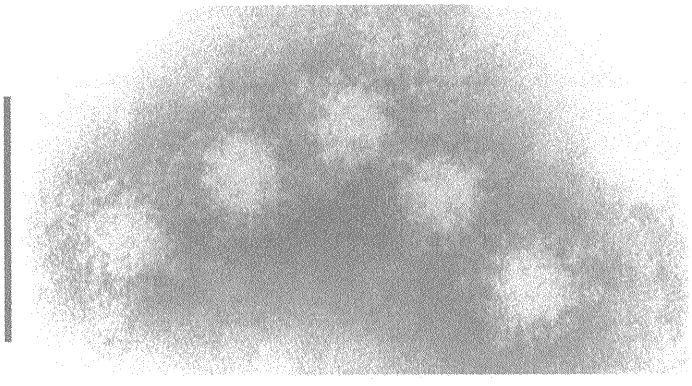


Figure 1: Negative contrast electron micrograph of virions of an isolate of hepatitis E virus, in the bile fluid from a monkey challenged with the genotype 2 Mexican strain of hepatitis E virus. The bar represents 100 nm. (From Ticehurst *et al.* (1992). *J. Infect. Dis.*, **165**, 835–845; with permission.)

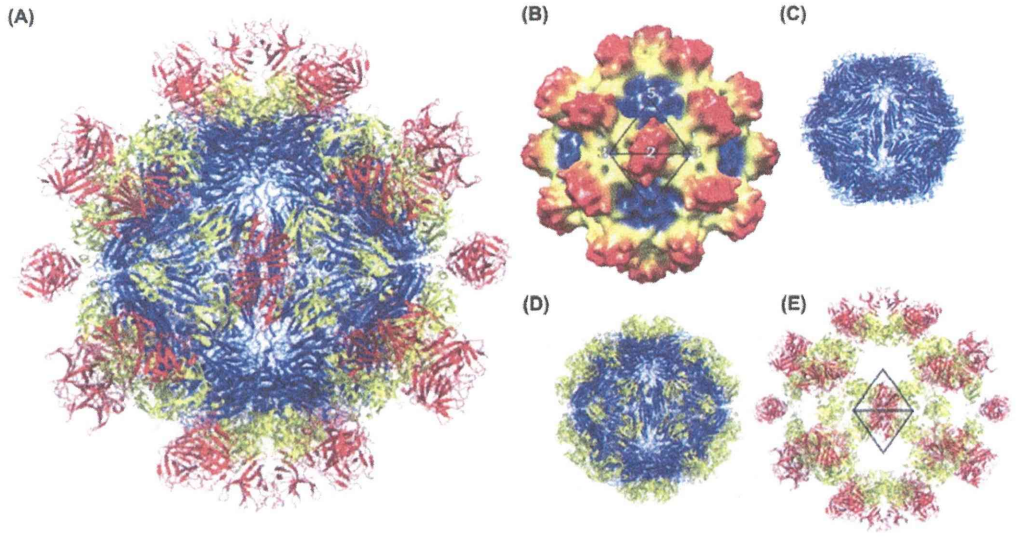


Figure 2: Structure of the hepatitis E virus-like particle (VLP) ($T = 1$). (A) Crystal structure of hepatitis E virus VLP. The three domains, S, P1 and P2 are colored blue, yellow and red, respectively. The VLP is positioned in a standard orientation with the 3 2-fold icosahedral symmetry axes aligned along the vertical, horizontal, and viewing directions, respectively. (B) Cryo-EM reconstruction at 14 Å resolution. The surface is colored by radial depth cue from blue, yellow, to red. (C) Hepatitis E virus VLP with only the S domain. (D) VLP with S and P1 domains. (E) VLP with P1 and P2 domains. (From Guu *et al.* (2009). *Proc. Natl Acad. Sci., U S A*, **106**, 12992–12997; with permission.)

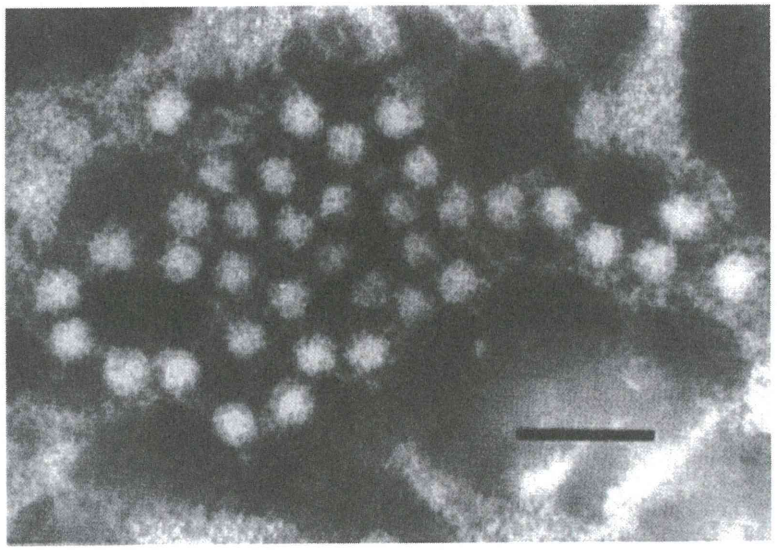


Figure 3: Electron micrograph of 30–35 nm diameter particles of Avian hepatitis E virus. The virus particles were detected from a bile sample of a chicken with hepatitis–splenomegaly syndrome. Bar = 100 nm. (From Haqshenas *et al.* (2001). *J. Gen. Virol.*, **82**, 2449–2462; with permission.)

Proteins

Virions are constructed from a major capsid protein encoded by the second open reading frame (ORF2), and the CP may be proteolytically processed. A small immunoreactive protein (12.5 kDa) encoded by the third ORF (ORF3) has been identified and shown to exhibit multiple functions associated with virion morphogenesis and viral pathogenesis. Non-structural proteins encoded by the

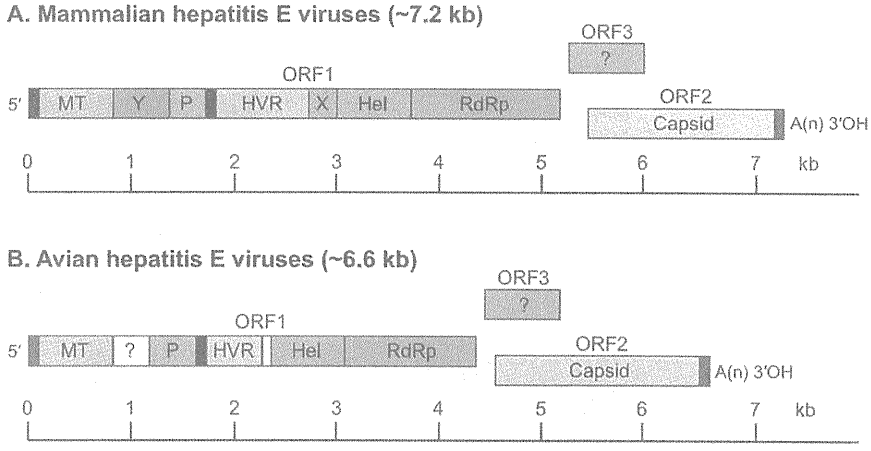


Figure 4: Schematic diagram of the genomic organization of hepatitis E virus: a short 5′ non-coding region (NCR), a 3′ NCR, and three ORFs. ORF2 and ORF3 overlap each other but neither overlaps ORF1. ORF1 encodes non-structural proteins including putative functional domains; ORF2 encodes capsid protein and ORF3 encodes a small phosphoprotein with a multi-functional C-terminal region. MT, methytransferase; Y, "Y" domain; P, a papain-like cystein protease; HVR, a hypervariable region that is dispensable for virus infectivity; Hel, helicase; RdRp, RNA-dependent RNA polymerase (From Meng, X.J. (2008). Hepatitis E virus (*Hepevirus*). In: *Encyclopedia of Virology* (5 vols), 3rd edn (B.W.J. Mahy and M.H.V. van Regenmortel, Eds.), Oxford, Elsevier, pp. 377–383; with permission.)

first major ORF (ORF1) have limited similarity with the "alpha-like supergroup" of viruses and contain domains consistent with a Mtr, RNA helicase, papain-like cysteine protease, and RdRp. The translational and posttranslational processes of the non-structural polyprotein remain unresolved. It remains unclear whether the non-structural polyprotein functions as a single protein with multiple functional domains or as individually-cleaved smaller proteins. RdRp, Mtr/guanylyltransferase and NTPase/RNA helicase activities have been experimentally demonstrated for ORF1 recombinant proteins.

Lipids

None reported.

Carbohydrates

Evidence for glycosylation of the major CP has been reported following its expression in mammalian cells. The CP sequence contains three potential sites for N-linked glycosylation and a signal peptide sequence at its N terminus. Mutations within the CP glycosylation sites prevent the formation of infectious virus particles, although the lethal effect is due to altered protein structure rather than elimination of glycosylation.

Genome organization and replication

The RNA genome of HEV is organized into three ORFs, with the non-structural proteins encoded toward the 5′ end of the genome and the structural protein(s) toward the 3′ end. Capped genomic RNA is infectious for chickens, pigs, rhesus monkeys and chimpanzees. The 5′-NCR is only about 26 nt long, and a cap structure has been identified in the 5′ end of the viral genome and may play a role in the initiation of hepatitis E virus replication. The 3′-NTR contains a *cis*-reactive element. ORF1 encodes the non-structural polyprotein. ORF2 encodes the major CP, and binds to cell surface heparan sulfate proteoglycans (HSPGs) in liver cells. The ORF3 encodes a small phosphoprotein (113–114 aa) with a multifunctional C-terminal region. ORF2 overlaps ORF3, but neither overlaps with ORF1 (Figure 4). A bicistronic subgenomic mRNA encoding both ORF2 and ORF3 proteins has been identified.

Although avian hepatitis E virus shares only about 50% nucleotide sequence identity with HEV isolates, the genomic organization and functional motifs are relatively conserved between them (Figure 4).

Antigenic properties

A single serotype of HEV has been described, with extensive cross-reactivity among circulating human and swine strains. Antibodies cross-reactive with capsid protein epitopes of human strains have been reported in various animal species but the viruses responsible for the cross-seropositivity were genetically identified only in pig, chicken, deer, mongoose, rat and rabbit. Avian hepatitis E virus also cross-reacts serologically with strains of HEV, and common antigenic epitopes have been identified in the capsid protein.

Biological properties

HEV is associated in humans with outbreaks and sporadic cases of enterically transmitted acute hepatitis. The virus is considered endemic in tropical and subtropical countries of Asia and Africa, as well as in Mexico, but antibody prevalence studies suggest a global distribution of this virus. HEV is a recognized zoonotic virus, and pigs and more likely other animal species are reservoirs.

Transmission is via the fecal–oral route. Sporadic cases of human hepatitis E have been reported in both industrialized and developing countries, although epidemics occur only in developing countries. The sources of infection appear to be different for epidemics and sporadic cases: contaminated drinking water is the main source for epidemics, whereas the risk factors for sporadic cases include shellfish, contaminated animal meats and direct contacts with infected animals. Human-to-human transmission seems rare in hepatitis E epidemics. Genotypes 1 and 2 strains are restricted to humans, whereas genotypes 3 and 4 strains have a broader host range and are zoonotic. Interspecies transmission of genotypes 3 and 4 hepatitis E virus between swine and non-human primates has been experimentally demonstrated. Pig handlers in both developing and industrialized countries are shown to be at increased risk of hepatitis E virus infection. Sporadic cases of acute hepatitis E have been epidemiologically and genetically linked to the consumption of contaminated raw and undercooked animal meats.

Avian hepatitis E virus infection in chickens is widespread and approximately 71% of chicken flocks and 30% of chickens in the United States were positive for IgG antibodies to the virus. Avian hepatitis E virus infected turkeys but failed to infect two rhesus monkeys, suggesting that the virus is likely not zoonotic. Attempts to experimentally infect mice and pigs with avian hepatitis E virus were unsuccessful. In chickens experimentally infected with avian hepatitis E virus, replicating viruses were detected in livers as well as in several extrahepatic tissues, indicating that the virus replicates not only in the liver but in the gastrointestinal tissues as well.

GENUS *HEPEVIRUS*

Type species ***Hepatitis E virus***

Distinguishing features

Viruses within the genus *Hepevirus* infect mammals, and thus far strains within this genus have been genetically identified from human, pig, mongoose, deer, rat and rabbit.

List of species in the genus *Hepevirus*

Hepatitis E virus		
Hepatitis E virus 1 (Burma)	[M73218]	(HEV-1)
Hepatitis E virus 2 (Mexico)	[M74506]	(HEV-2)
Hepatitis E virus 3 (Meng)	[AF082843]	(HEV-3)
Hepatitis E virus 4 (T1)	[AJ272108]	(HEV-4)

Species names are in italic script; names of strains are in roman script. Sequence accession numbers [ ] and assigned abbreviations ( ) are also listed.

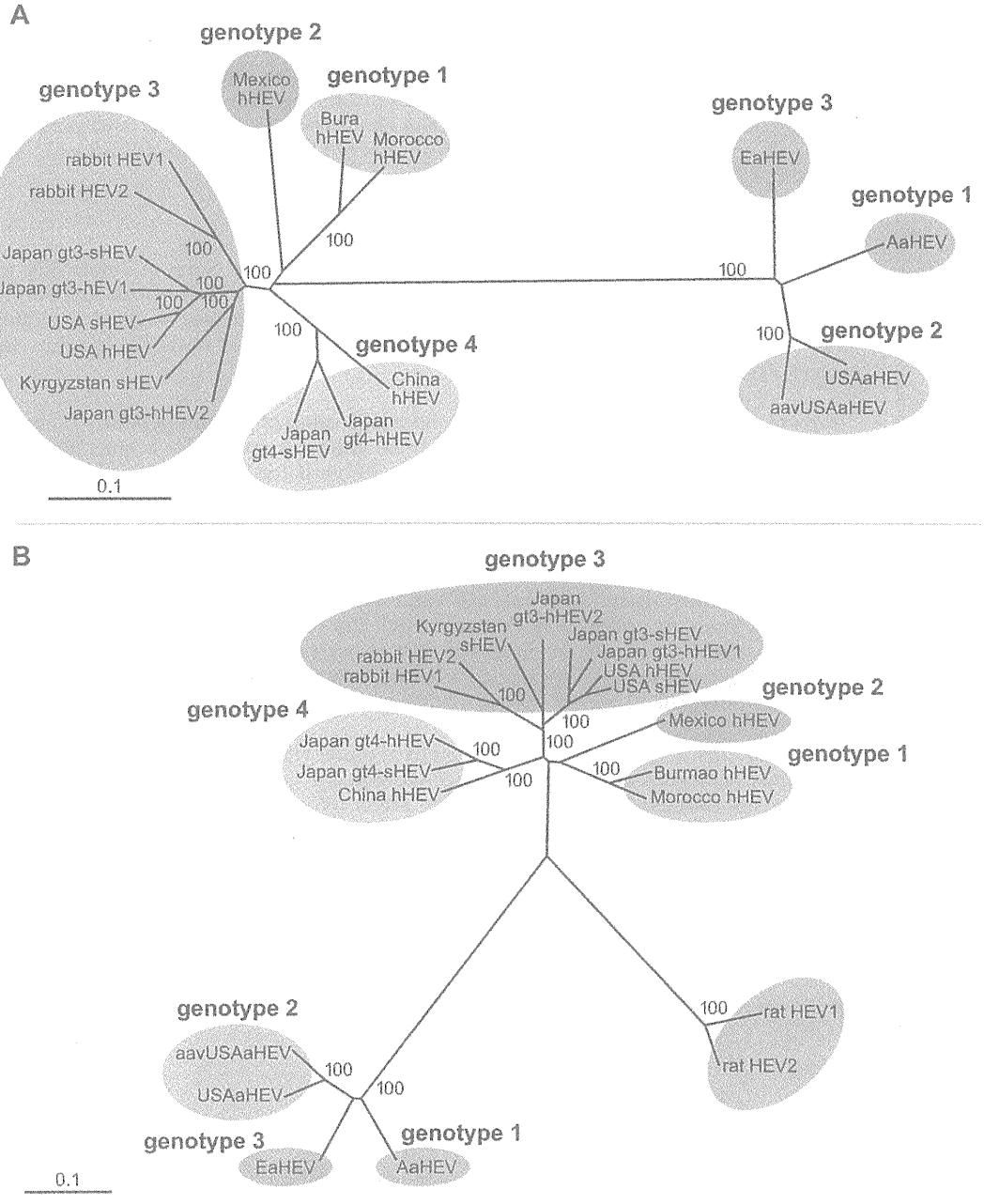


Figure 5: Phylogenetic trees depicting the relationship between strains of mammalian hepatitis E virus in the genus *Hepevirus* and the unassigned species *Avian hepatitis E virus* (courtesy of Hiroaki Okamoto). (A) A phylogenetic tree based on the full-length genomic sequences of representative hepatitis E virus strains including the four major genotypes of mammalian hepatitis E virus, the newly-identified rabbit hepatitis E virus and the three genotypes of Avian hepatitis E virus; (B) A phylogenetic tree based upon partial sequence (1545 nt) of the rat hepatitis E virus along with other hepatitis E virus strains in Figure 6A. GenBank accession numbers for the strains used in these analyses are Burma hHEV (M73218); Morocco hHEV (AY230202); Mexico hHEV (M74506); USA hHEV (AF060669); USA sHEV (AF082843); Japan gt3-hHEV1 (AP003430); Japan gt3-sHEV (AB073912); Japan gt3-hHEV2 (AB248520); Kyrgyzstan sHEV (AF455784); China hHEV (AJ272108); Japan gt4-sHEV (AB097811); Japan gt4-hHEV (AB220973); USAaHEV (AY535004); aavUSAaHEV (EF206691); AaHEV (AM943647); EaHEV (AM943646); rabbit HEV1 (FJ906895); rabbit HEV2 (FJ906896); rat HEV1 (GQ504009); and rat HEV2 (GQ504010).

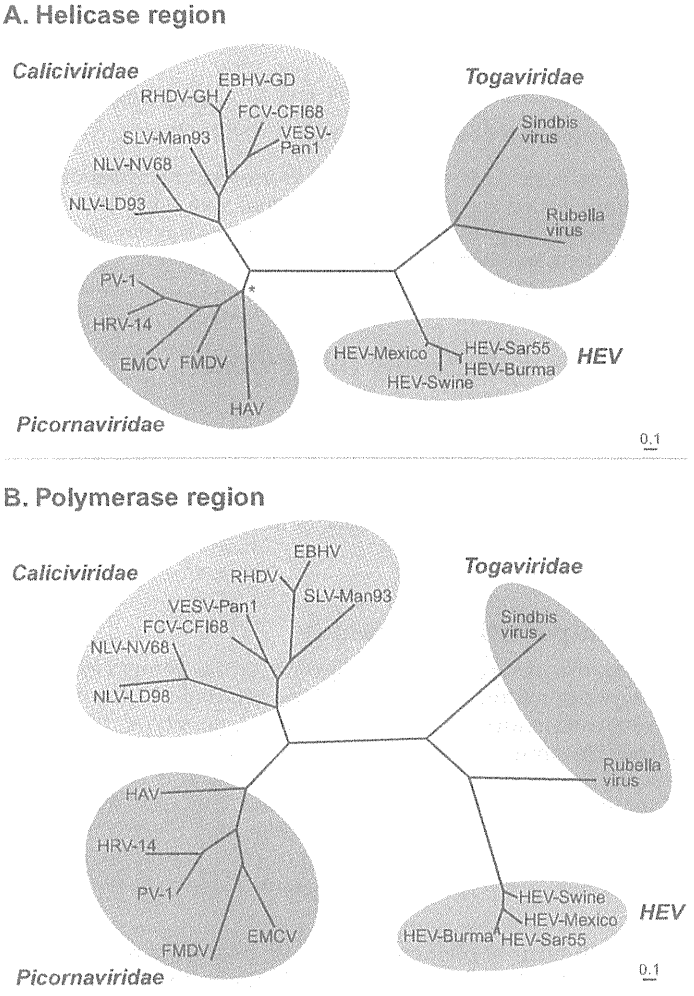


Figure 6: Phylogenetic relationships of hepatitis E virus with members of the families *Picornaviridae*, *Caliciviridae* and *Togaviridae*. The helicase (Hel) and polymerase (Pol) regions of the genome were analyzed (courtesy of T. Berke and D.O. Matson). (A) Partial gene sequences (200 aa) from the proposed helicase region were used for the phylogenetic analysis and included representative strains from each family. Clustal W v1.7 was used to create a multiple alignment for the aa sequences, which was verified by alignment of known motifs in the region (e.g. GxGKS/T). The nt sequences were added and aligned by hand using the corresponding aa sequences as a template resulting in a consensus length of 608 nt. A phylogenetic tree was constructed from the nt sequence alignment using the maximum likelihood algorithm in the program DNAML from the PHYLIP 3.52c package within UNIX environment. For the algorithm, the global rearrangement option was invoked and the order of sequence input was randomized ten times. Other menu options were left as default. The resultant tree is unrooted and the phylogenetic distances are in the unit of expected number of substitutions per site. Branch points of the resulting tree had a confidence level of $P<0.01$ ($P<0.05$*). GenBank accession numbers for the strains in this analysis were M87661 (Norwalk virus, NLV-NV68), X86557 (Lordsdale virus, NLV-LD93), U52086 (primate calicivirus, VESV-Pan1), U13992 (feline calicivirus, FCV-CFI/68), Z69620 (European brown hare syndrome virus, EBHV-GD), M67473 (rabbit hemorrhagic disease virus, RHDV-GH), X86560 (Sapporo virus, SLV-Man93), J02281 (human poliovirus 1, PV-1), K02121 (Human rhinovirus type 14, HRV-14), M22458 (encephalomyocarditis virus, EMCV), X00429 (foot-and-mouth disease virus, FMDV), K02990 (hepatitis A virus, HAV), M15240 (rubella virus), J02363 (Sindbis virus), M73218 (hepatitis E virus, HEV-Burma), M80581 (hepatitis E virus, HEV-Sar55), M74506 (hepatitis E virus, HEV-Mexico), AF011921 (hepatitis E virus, HEV-Swine). (B) Partial gene sequences (200 aa) from the proposed polymerase region were used for the phylogenetic analysis and included representative strains from each family. Clustal W v1.7 was used to create a multiple alignment for the aa sequences, which was verified by alignment of known motifs in the region (e.g. SGxxxTxxxMT/S, GDD). The nt sequences were added and aligned by hand using the corresponding aa sequences as template resulting in a consensus length of 590 nt. A phylogenetic tree was constructed as described above and GenBank accession numbers for the strains in this analysis were identical to those above.