ORF3 protein of hepatitis E virus is essential for virion release from infected cells

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The function of the hepatitis E virus (HEV) open reading frame 3 (ORF3) protein remains unclear. To elucidate the role of the ORF3 protein in the virus life cycle, an infectious cDNA clone (pJE03-1760F/wt) that can replicate efficiently in PLC/PRF/5 and A549 cells and release progeny into the culture medium was used to generate a derivative ORF3-deficient (ΔORF3) mutant whose third in-frame AUG codon of ORF3 was mutated to GCA. The ΔORF3 mutant in the culture medium of mutant RNA-transfected PLC/PRF/5 cells was able to infect and replicate within PLC/PRF/5 and A549 cells as efficiently as the wild-type pJE03-1760F/wt virus. However, less than 1/100 of the number of progeny was detectable in the culture medium of ΔORF3 mutant-infected PLC/PRF/5 cells compared with wild-type-infected PLC/PRF/5 cells, and the HEV RNA level in the culture medium of ΔORF3 mutant-infected A549 cells was below or near the limit of detection. An immunocapture PCR assay revealed that the ORF3 protein is present on the surface of cell-culture-generated wild-type HEV but not on the ΔORF3 mutant. Wild-type HEV in the culture supernatant peaked at a sucrose density of 1.15–1.16 g ml⁻¹, in contrast with the ΔORF3 mutant in culture supernatant, which banded at 1.27–1.28 g ml⁻¹, similar to HEV in cell lysate and faecal HEV. These results suggest that the ORF3 protein is responsible for virion egress from infected cells and is present on the surface of released HEV particles, which may be associated with lipids.

INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of acute hepatitis E, which occurs in many parts of the world (Emerson & Purcell, 2006; Harrison, 1999; Okamoto et al., 2003; Worm et al., 2002). Several species of animal, such as swine, are considered to serve as reservoirs of HEV (Meng et al., 1997; Takahashi et al., 2003), and ingestion of uncooked or undercooked meat and 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 is classified into four major genotypes. Genotypes 1 and 2 have caused outbreaks of hepatitis E as water-borne epidemics, whilst genotypes 3 and 4 have been found in sporadic cases of acute hepatitis E that were most likely zoonotic in origin (Okamoto, 2007).

HEV is the sole member of the genus Hepevirus in the family Hepeviridae (Emerson et al., 2005). HEV is a non-enveloped virus and its genome is a single-stranded, positive-sense RNA, which is capped and polyadenylated (Kabrane-Lazizi et al., 1999; Tam et al., 1991). The genome is approximately 7.2 kb and contains three open reading frames (ORF1–3) (Tam et al., 1991). ORF1 encodes non-structural proteins including the helicase and 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 (Graff et al., 2006). The ORF2 protein is the viral capsid protein, whilst the ORF3 protein is a small protein whose function(s) has not been fully defined.

Recently, we developed an efficient cell-culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5) and a lung cancer cell line (A549) using a genotype 3 HEV (JE03-1760F strain) obtained from a faecal specimen of a Japanese patient with hepatitis E (Tanaka et al., 2007). In this system, consecutive passages of culture supernatant were successfully carried out in PLC/PRF/5 and A549 cells, with the highest HEV load reaching 10⁸ copies ml⁻¹ in the culture medium (Lorenzo et al., 2008). More recently, we...
established an infectious cDNA clone of the JE03-1760F strain and found that the JE03-1760F cDNA (pJE03-1760F/wt)-generated virus could infect and replicate as efficiently as the native, wild-type virus in cell culture (Yamada et al., 2009).

It has been reported that 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, z1-microglobulin/bikunin precursor, tumour susceptibility gene 101 (Tsg101) and the src homology 3 domains (Korkaya et al., 2001; Surjit et al., 2006; Tyagi et al., 2004; Zafrrullah et al., 1997). However, it remains unclear whether the reported functions of ORF3 protein are authentic in the HEV life cycle and whether ORF3 protein is associated with virion morphogenesis. Recent studies using infectious HEV cDNA clones have suggested that intact ORF3 protein expression is essential for infection of animals (Graff et al., 2005; Huang et al., 2007), but is not required for infection and virion morphogenesis in vitro (Emerson et al., 2006). Our recent immunocapture PCR assay using a monoclonal antibody (mAb) against ORF3 protein revealed the presence of ORF3 protein on the surface of cell-culture-generated HEV (Takahashi et al., 2008b). Therefore, one critical function of the ORF3 protein may be associated with virus egress from infected cells (Emerson et al., 2006). In the present study, we generated a derivative ORF3-deficient (ΔORF3) mutant and a mutant with insertion of a T between the second and third in-frame ATG codons of ORF3, and examined whether ORF3 protein is responsible for virion morphogenesis and release of virus from infected cells.

**METHODS**

**Virus.** A fecal suspension containing a high load of wild-type HEV (JE03-1760F strain: 2.0 x 10⁸ copies ml⁻¹) (Tanaka et al., 2007) was used as the reference in this study.

**Cells.** PLC/PRF/5 (ATCC CRL-8024) and A549 (ATCC CCL-185) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 U penicillin G ml⁻¹, 100 µg streptomycin ml⁻¹ and 2.5 µg amphotericin B ml⁻¹ (growth medium), at 37 °C in a humidified 5% CO₂ atmosphere, as described previously (Tanaka et al., 2007).

**Plasmid construction.** With the advent of a full-length infectious cDNA clone of the JE03-1760F strain (pJE03-1760F/wt) (Yamada et al., 2009), two recombinant plasmids containing JE03-1760F mutant genomes, named pJE03-1760F/ΔORF3 (AORF3) and pJE03-1760F/ΔF, were constructed using a QuickChange II Site-Directed Mutagenesis kit (Stratagene) with primers ΔORF3-f and ΔORF3-r or fs-f and fs-r (Supplementary Table S1, available in JGV Online), respectively. As depicted in Fig. 1, AORF3 had nucleotide mutations of ATG to GCA at the third in-frame methionine codon of ORF3, and pJE03-1760F/ΔF had an insertion of a T between the second and third in-frame ATG codons of the ORF3. Construction of the two recombinant plasmids was verified by sequence analysis as described previously (Takahashi et al., 2007). As a negative control, an ORF1-deficient mutant genome (pJE03-1760F/AORF1) containing a frameshift mutation in ORF1 (Yamada et al., 2009) was also used.

**In vitro transcription and transfection in PLC/PRF/5 cells.** Each of the four full-length genome plasmids described above was linearized with NheI. Using 2 µg of each of the linearized plasmids as template, a genomic RNA was transcribed with an AmpliScribe T7-Flash Transcription kit (EPICENTRE Biotechnologies). After in vitro transcription, RNA transcripts of all four cDNA clones were capped using a ScriptCap m7G Capping System (EPICENTRE Biotechnologies). The size and yield of the transcripts were observed by electrophoresis on a MOPS/formaldehyde agarose gel. Three micrograms of capped RNA was transfected into subconfluent (60–80% confluent) PLC/PRF/5 cells in a well of a six-well plate (Iwaki) in duplicate, using a TransIT-mRNA transfection kit (Mirus Bio) and then incubated at 37 °C. At 2 days post-transfection (p.t.), the culture medium was replaced with 2 ml growth medium and incubated at 35.5 °C. Every other day, half of the culture medium (1 ml) was replaced with fresh maintenance medium consisting of 50% DMEM and 50% medium 199 (Invitrogen) containing 2% FCS, 30 mM MgCl₂ and antibiotics. The collected medium was centrifuged at 800 g at 4 °C for 10 min and the supernatant was stored at −80 °C until use.

**Virus inoculation.** Monolayers of PLC/PRF/5 or A549 cells in a six-well plate were washed with PBS; 0.2 ml culture supernatant of the transfected cells, which had been filtered through a 0.22 µm pore size microfilter and then diluted with PBS containing 0.2% (w/v) BSA, was inoculated onto the cells. After inoculation at room temperature for 1 h, the cells were washed with PBS and 2 ml maintenance medium was added. The inoculated cells were incubated at 35.5 °C, and every other day, half of the culture medium (1 ml) was replaced with fresh maintenance medium, and the harvested medium was centrifuged at 800 g at 4 °C for 10 min and the supernatant was stored at −80 °C until virus titration.

For quantification of extracellular and intracellular HEV RNAs, culture medium was collected from each well and centrifuged at 800 g at 4 °C for 10 min and the supernatant was stored at −80 °C until use. After removing as much of the culture medium as possible, the inoculated cells in each well were washed with PBS, trypsinized, pelleted by centrifugation and suspended in maintenance medium. After counting the number of cells, the medium was centrifuged and the resulting cell pellets stored at −80 °C.

Cell lysates were prepared as follows. Monolayers of transfected or infected cells in the six-well plate were collected by scraping and centrifuged at 100 g at 4 °C for 5 min and the supernatant was then removed from the cell pellet. The cell pellet was resuspended in maintenance medium and lysed by three freeze–thaw cycles. The debris was removed by centrifugation at 1500 g at 4 °C for 10 min and the supernatant was stored at −80 °C. The lysate was thawed and centrifuged at 13,000 g at 4 °C for 5 min and the supernatant was filtered through a 0.22 µm microfilter.

**Quantification of HEV RNA.** HEV RNA was quantified by two distinct real-time RT-PCR methods, using two sets of primers and a probe. RNA extractions from culture supernatants and from infected cells (cell pellets) were performed using TRIzol-LS and TRIzol (Invitrogen), respectively. The extracted RNA was subjected to real-time RT-PCR with a Quant iTect Probe RT-PCR kit (Qiagen) in a LightCycler apparatus (Roche Diagnostics). To target the ORF2 and ORF3 overlapping region, sense and antisense primers and a probe consisting of an oligonucleotide with a 5’ reporter dye (FAM) and a 3’ quencher dye (TAMRA) were used in a real-time RT-PCR assay as described previously (Takahashi et al., 2008a). In addition, to target the 5’-terminal region of the ORF1 gene, newly designed primers HE447 and HE448 and a probe (HE446-P) (Supplementary Table S1) were used. Thermal cycler conditions were the same in the two real-time RT-PCR methods, and consisted of 50 °C for 20 min, 95 °C for 15 min, 45 cycles of 95 °C for 0 s and 60 °C for 60 s. Unless
otherwise stated, quantification of HEV RNA was performed using the real-time RT-PCR method with the probe targeting the ORF2/ ORF3 region.

**Immunofluorescence assay (IFA).** Transfected cells were fixed with 4 % paraformaldehyde for 30 min and permeabilized with 0.2 % Triton X-100 at room temperature for 10 min. After washing with PBS, the fixed cells were incubated with a mAb against the ORF2 protein (H6225) (Takahashi et al., 2008a) or an anti-HEV ORF3 mAb (TA0536) (Takahashi et al., 2008b) at 37 ºC for 1 h. After washing with PBS, the cells were stained with Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) at 37 ºC for 1 h. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Roche Diagnostics). The stained cells were observed under a BX-8000 digital microscope (Keyence) and the images were processed by using BZ-Analyzer software (Keyence).

**Western blotting.** Transfected cells were lysed in lysis buffer [50 mM Tris/HCl (pH 8.0), 1 % NP-40, 150 mM NaCl] and the lysed cells were mixed with an equal volume of 2 × SDS-PAGE sample buffer [125 mM Tris/HCl (pH 6.8), 4 % SDS, 10 % (w/v) sucrose, 10 % (v/v) 2-mercaptoethanol, 0.01 % (w/v) bromophenol blue]. After incubation at 95 ºC for 5 min, proteins in the samples were separated by SDS-PAGE using an 8 or 15 % gel and transferred to a PVDF membrane (0.45 µm; Millipore) for analysis by Western blotting. The membrane was incubated with anti-HEV ORF2 mAb (H6210) (Takahashi et al., 2008a) or anti-HEV ORF3 mAb (TA0536) in PBS containing 2.5 % skimmed milk and 0.1 % Tween 20 at 37 ºC for 1 h. After washing with PBS containing 0.1 % Tween 20, the membrane was incubated with ECL horseradish peroxidase-linked anti-mouse IgG (from sheep (diluted 1:20 000; GE Healthcare)). Protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

**Virus adsorption assay.** A virus adsorption assay was performed as follows. After washing A549 cells grown in a six-well plate (3.5–4.0 × 10⁶ cells per well) with PBS, virus (10⁴ or 10⁵ copies) in culture supernatant or cell lysate was inoculated onto monolayers of the cells. After incubation at room temperature for 1 h, the cells were washed with PBS, resuspended in PBS and collected by centrifugation at 800 g at 4 ºC for 10 min. After removal of the supernatant, the cell pellet was stored at −80 ºC until virus titration.

**Immunocapture RT-PCR assay.** The immunocapture RT-PCR assay was performed as described previously (Takahashi et al., 2008a). Briefly, anti-ORF2 mAb (H6225), anti-ORF3 mAb (TA0536) and mAb 5520 raised against hepatitis B virus pre-S2 protein (Okamoto et al., 1985) as a negative control were used in this assay. These mAbs were biotinylated and purified by a method described previously (Takahashi et al., 2008b). The wells of a Reacti-Bind Streptavidin High Binding Capacity Coated Plate (Thermo Scientific) were washed with saline and 100 µl biotinylated mAb (1 µg ml⁻¹) in PBS containing 0.1 % BSA and 0.2 % Tween 20 was added to each well. The wells were incubated with gentle shaking at room temperature for 1 h and then washed with saline. One hundred microlitres of each virus sample was added to each well and incubated at room temperature for 2 h and then at 4 ºC overnight. The solution in each well was removed and the wells were washed with saline. The RNA in each well was then extracted with TRIzol-LS reagent and subjected to quantitative detection of HEV RNA. In addition, to examine whether prior treatment with bile would affect the binding efficiency of mAbs with HEV particles, 10 µl culture supernatant or faecal suspension was mixed with 100 µl bile, incubated at 37 ºC for 2 h and diluted 1:100 with PBS containing 0.1 % BSA and then subjected to an immunocapture RT-PCR assay as described above. A bile sample was obtained from a patient with severe cholestasis who was receiving bile drainage, and stored at −80 ºC (Ukita et al., 1999).

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**RESULTS**

**Construction of full-length cDNA clones of HEV JE03-1760F mutants**

As shown in Fig. 1, the full-length cDNA clone of an ORF3-deficient mutant (ΔORF3) was constructed by mutating the third in-frame start codon of the ORF3 gene (ATG→GCA) in the full-length cDNA of the wild-type strain JE03-1760F, based on a previous report that the third in-frame initiation codon is necessary for ORF3 protein synthesis (Graff et al., 2006; Huang et al., 2007). To confirm that the first and second in-frame AUG codons of ORF3 in HEV genotypes 1–3 are dispensable for ORF3 protein expression, we generated an additional mutant clone, pJE03-1760F/fs, in which a T was inserted between the second and third in-frame AUG codons of the JE03-1760F genome (Fig. 1), consistent with all reported genotype 4 HEV strains (Inoue et al., 2006; Nishizawa et al., 2003; Takahashi et al., 2002; Wang et al., 2000). The size and quantity of the RNA transcripts generated were almost equal among the four genome plasmids for the wild type (pJE03-1760F/wt) and its three mutants (ΔORF3, pJE03-1760F/fs and pJE03-1760F/ΔORF1) (data not shown).

**Insertion of a U between the second and third in-frame AUGs of the ORF3 gene of the HEV genome does not influence HEV viral protein expression and replication in cultured cells**

With regard to HEV protein expression and viral replication, there were no discernible differences between the wild-type pJE03-1760F/wt and the mutant pJE03-1760F/fs. As with transfection of the wild-type clone, both ORF2 and ORF3 proteins were expressed in PLC/PRF5 cells that had been transfected with pJE03-1760F/fs RNA, as demonstrated by IFA at 8 days p.t. (Fig. 2a) and Western blotting at 60 days p.t. (Fig. 2b). The migration patterns of the ORF2 and ORF3 proteins (83 and 13 kDa, respectively) on SDS-PAGE were indistinguishable between the wild type and pJE03-1760F/fs (Fig. 2b, lanes 1 and 3). The HEV RNA level in the culture supernatant of pJE03-1760F/fs RNA-transfected cells increased in almost the same manner.
as that of the wild-type RNA-transfected cells. A gradual decrease in HEV RNA level was observed in the culture medium of the ΔORF1 mutant RNA-transfected cells, which served as a negative control, whose titre probably reflects residual amounts of the introduced RNA transcripts in the culture medium (Fig. 2c). Sequence analysis of the JE03-1760F/fs genome recovered from the culture supernatant at 60 days p.t. confirmed the presence of an insertion of a U after nt 5146 and the absence of any undesired mutation within the entire ORF3 gene and its upstream region (data not shown). Even on passages of culture supernatant, the changing pattern of virus production over time in the culture supernatant of PLC/PRF/5 and A549 cells was almost identical between pJE03-1760F/wt and pJE03-1760F/fs (Fig. 3).

**Lower levels of HEV RNA in the culture supernatant of ΔORF3 mutant RNA-transfected cells than with wild-type genome RNA-transfected cells**

As expected, ORF3 protein expression was not detectable by IFA in PLC/PRF/5 cells transfected with RNA transcripts of ΔORF3 mutant, despite the clearly detectable expression of the ORF2 protein (Fig. 2a). Western blot analysis revealed expression of the ORF2 protein but not the ORF3 protein in the ΔORF3 mutant RNA-transfected cells at 60 days p.t. (Fig. 2b, lane 2). The HEV RNA level in the culture supernatant of the ΔORF3 mutant RNA-transfected cells increased gradually from 16 days p.t., reaching $5 \times 10^6$ copies ml$^{-1}$ at 60 days p.t., although the level was approximately 10-fold lower than that of cells transfected with the wild-type RNA during the observation period of 16–60 days p.t. (Fig. 2c). Nucleotide sequence analysis of the propagated ΔORF3 mutant in the culture supernatant at 60 days p.t. confirmed the presence of the GCA mutation at the third in-frame AUG codon of the ORF3 gene and the absence of any other mutations in the entire ORF3 gene and its upstream region (data not shown).

**ORF3 protein is required for viral egress from infected cells**

To examine whether the ΔORF3 mutant virus in the culture medium of the transfected cells was infectious and replication-competent and, if so, whether HEV virions were released from ΔORF3 mutant-infected cultured cells, the culture supernatant of the transfected cells was inoculated into three wells each of PLC/PRF/5 or A549 cells at an HEV load of $10^5$ and $10^4$ copies per well.
respectively. Of interest, viral RNA was almost undetectable in the culture supernatant of A549 cells inoculated with the ΔORF3 mutant up to the end of the observation period [30 days post-inoculation (p.i.)] (Fig. 3). For the PLC/PRF/5 cells inoculated with the ΔORF3 mutant, less than 1/100 of the number of progeny was detectable in the culture medium compared with pJE03-1760F/wt. Sequence analysis of the region flanking the introduced mutation at 30 days p.i. excluded the possibility that the virus underwent a reversion to complement the deleterious mutation (data not shown). 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 detected at 30 days p.i. at a titre of $5.8 \times 10^5$.
and 2.3 × 10^4 copies per well (geometric mean, n=3) in PLC/PRF/5 and A549 cells, respectively, and was almost equal to titres in the respective cells inoculated with pJE03-1760F/wt.

To verify these observations, we determined the intra- and extracellular viral RNA levels in ΔORF3 mutant-inoculated A549 cells at 0, 8, 16 and 24 days p.i. and compared them with those in pJE03-1760F/wt-inoculated cells, using two real-time RT-PCR methods with probes targeting distinct genomic regions. Supporting the above-mentioned results (Fig. 3), extracellular viral RNA was not detected in the culture medium of ΔORF3 mutant-infected A549 cells, whereas the extracellular viral RNA level in the culture medium of wild-type-infected A549 cells increased with time (Fig. 4a, b), regardless of the target region for HEV RNA quantification. Nevertheless, the intracellular viral RNA level increased with time in both the pJE03-1760F/wt-infected and ΔORF3 mutant-infected cells, and was almost equal in the two infected cell types at 16 and 24 days p.i. (Fig. 4c, d). In addition, the ratio of the HEV RNA titre detectable by the ORF2/3 probe, which is capable of detecting both genomic and subgenomic RNAs (ORF2/3 RNA), to that detectable by the ORF1 probe, which is capable of detecting only genomic RNA (ORF1 RNA), was calculated for the samples at 24 days p.i. in triplicate. The ratio of ORF2/3 RNA to ORF1 RNA in the culture supernatant of pJE03-1760F/wt-infected cells was estimated to be 1.16 ± 0.09 (mean ± SD), which was similar to those of

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**Fig. 3.** Quantification of HEV RNA in culture supernatants of PLC/PRF/5 and A549 cells inoculated with the indicated viruses (in triplicate) and cultured for up to 30 days. Quantification was performed by real-time RT-PCR using a probe targeting the ORF2/ORF3 region.
in vitro-transcribed full-length RNA (1.05 ± 0.08) and faecal RNA (1.09 ± 0.09), indicating that the two probes have almost the same sensitivity with regard to detection of genomic RNA and that virions released from infected cells may not possess subgenomic RNA. The ratios of intracellular ORF2/3 RNA to ORF1 RNA were quite similar in pJE03-1760F/wt-infected and ΔORF3 mutant-infected A549 cells (4.18 ± 0.94 and 4.41 ± 0.82, respectively), suggesting that the ratio of subgenomic RNA synthesis to genomic RNA synthesis is not affected by the absence of the ORF3 protein.

**Adsorption efficiency of wild-type HEV and ΔORF3 mutant**

Although the HEV viral load inoculated in each well (10^5 copies per well) was exactly the same and the number of cells in each well was comparable between the pJE03-

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**Fig. 4.** Levels of intracellular and extracellular HEV RNA in infected cells. (a–d) A549 cells were inoculated at 10^5 copies per well with culture supernatant of pJE03-1760F/wt RNA- or ΔORF3 RNA-transfected PLC/PRF/5 cells. The infected cells and their supernatants were harvested at 0, 8, 16 and 24 days p.i. The HEV viral load in culture supernatants (a, b) and cells (c, d) was measured by real-time RT-PCR with primers and a probe targeting the ORF1 region (a, c) or the ORF2 and ORF3 overlapping region (b, d). (e) The number of harvested cells at 0, 8, 16 and 24 days p.i. Data are represented as the mean ± SD of values in three wells each.
1760F/wt-infected and ΔORF3 mutant-infected cells (Fig. 4e), the level of HEV RNA recovered from the inoculated cells at exactly 30 min p.i. (i.e. 0 days p.i.) was quite different, being below the detection limit in the wild-type inoculation and approximately 10^5 copies per 10^6 cells in the ΔORF3 inoculation (Fig. 4c, d). These results prompted us to compare the efficiency of adsorption to A549 cells between the wild-type virus and ΔORF3 mutant. Following inoculation of the culture supernatant of the ΔORF3 mutant RNA-transfected cells, the virus adsorbed to A549 cells in a dose-dependent manner, and 9.6 and 15% of the input virus bound to the cells following inoculation of 10^4 or 10^5 copies, respectively (Table 1). After inoculation of the culture supernatant of pJE03-1760F/wt RNA-transfected cells, adsorbed virus was detected only after inoculation of 10^5 copies at 2.6 × 10^5 copies per well, and the amount of adsorbed virus was 5.8-fold less than that of the ΔORF3 mutant following inoculation of 10^5 copies. In contrast, 14% of the input virus bound to the cells when the cell lysate from pJE03-1760F/wt-infected cells was added, and this binding efficiency was similar to that of the culture supernatant of the ΔORF3 mutant RNA-transfected cells rather than that of the culture supernatant of the pJE03-1760F/wt RNA-transfected cells.

Characterization of HEV particles in the culture supernatant and cell lysate

To examine whether the ORF3 protein associates with viral particles, an immunocapture RT-PCR assay was performed (Table 2). In the faeces-derived wild-type JE03-1760F and ΔORF3 mutant in the culture supernatant from transfected cells, although 93.2–96.6% of viral particles were captured by anti-ORF2 mAb, no virus was captured by anti-ORF3 mAb. In contrast, when the pJE03-1760F/wt and pJE03-1760F/fs viral particles in the culture supernatant of the transfected cells were tested, they were both captured by anti-ORF2 and anti-ORF3 mAbs, although only partially (~30%). Of note, treatment with bile resulted in the 30% binding by anti-ORF3 mAb being abolished, whilst the binding efficiency of the pJE03-1760F/wt and pJE03-1760F/fs viral particles in the culture supernatant by anti-ORF2 mAb increased to 79.3–83.4%.

The culture supernatant of pJE03-1760F/wt RNA-transfected cells and a faecal suspension containing the JE03-1760F strain as well as the culture supernatant and lysate of the ΔORF3 mutant RNA-transfected cells were subjected to ultracentrifugation in a sucrose density gradient. As for the wild-type virus, viral RNA in the culture supernatant banded at a density of 1.15–1.16 g ml⁻¹, which was markedly lower than that in faeces at 1.27–1.28 g ml⁻¹. Of note, HEV in the culture supernatant and lysate of the ΔORF3 mutant RNA-transfected cells peaked at 1.26–1.27 g ml⁻¹ (Fig. 5).

DISCUSSION

This study demonstrated that the mutant pJE03-1760F/fs whose genome has insertion of a U between the second and third in-frame AUG codons of ORF3, similar to all reported genotype 4 HEV strains, expressed both ORF2 and ORF3 proteins (Fig. 2). The molecular size of the ORF3 protein produced by pJE03-1760F/fs was indistinguishable from that of the ORF3 protein produced by the wild type by Western blotting. The size of the ORF2 protein produced by pJE03-1760F/fs was also unchanged. We also showed that ORF3 protein expression was abolished by the introduction of mutations in the third in-frame AUG codon of ORF3 (ΔORF3; Fig. 2). These results provide evidence that the third in-frame AUG codon is the authentic ORF3 start codon in the HEV replication cycle, corroborating previous reports (Graff et al., 2006; Huang et al., 2007). In addition, pJE03-1760F/fs showed the same replication property in cultured cells as the wild type, consistent with the report by Huang et al. (2007) who showed that introduction of the same U insertion in the swine HEV genome did not affect virus infectivity in pigs.

Table 1. Adsorption of wild-type HEV and ΔORF3 mutant to A549 cells

Monolayers of A549 cells (3.5–4.0 × 10^6 cells per well) were incubated with 10^4 or 10^5 copies of each virus for 1 h. The amount of adsorbed virus was measured by quantitative RT-PCR targeting the ORF2/3 region. Values represent the mean copies per well ± SD (n=3). Reproducibility was confirmed by repeated experiments.

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<tr>
<th>Inoculum*</th>
<th>Amount of adsorbed virus following introduction of:</th>
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<tr>
<td></td>
<td>10^4 copies</td>
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<tr>
<td>ΔORF3 (culture medium)</td>
<td>9.6 × 10^4 ± 1.3 × 10^2</td>
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<tr>
<td>pJE03-1760F/wt (culture medium)</td>
<td>&lt;2 × 10^4†</td>
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<tr>
<td>pJE03-1760F/wt (cell lysate)</td>
<td>1.4 × 10^4 ± 0.3 × 10^3</td>
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*Inocula were derived from the culture supernatant of transfected cells (pJE03-1760F/wt and ΔORF3) and the lysate of pJE03-1760F/wt-infected cells, as indicated.
†Under the detection limit (200 copies per well).
‡ND, Not done, due to unavailability of cell lysate containing HEV at 10^5 copies per 200 µl.
It has been reported that an infectious ORF3-null mutant virus could be extracted from cells by artificial cell disruption (Emerson et al., 2006). We often observed cell disruption 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 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, different from the wild-type virus in the culture supernatant and with lower efficiency (Table 1), and when applied to sucrose density ultracentrifugation, similar banding patterns peaking at 1.27–1.28 g ml\(^{-1}\) were observed in the culture supernatant and lysate of the ΔORF3 mutant RNA-transfected PLC/PRF/5 cells (Fig. 5). These results support the notion that the ΔORF3 mutant in the culture supernatant of the transfected or infected PLC/PRF/5 cells may be mostly intracellular virus. Therefore, it seemed likely that detection 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 could be ascribed to the artificial release of intracellular virus by cell disruption. Our previous studies revealed that not only PLC/PRF/5 cells but also A549 cells are permissive for HEV infection and propagation in a culture system using the JE03-1760F strain of faecal origin (Tanaka et al., 2007). Of interest, cell disruption was less frequently observed in A549 cells than in PLC/PRF/5 cells during long-term cell culture. In the present study, we found that HEV release into the culture supernatant was rarely detected in the ΔORF3 mutant-infected A549 cells (Figs 3 and 4), in which intracellular viral RNA was detected at levels similar to those in the virus-producing, wild-type-infected A549 cells (Fig. 4). These results confirmed the assumption proposed by Emerson et al. (2006) that the ORF3 protein is required for virion release from infected cells but not for replication, virion assembly or infection of culture cells.

Consistent with our previous study (Takahashi et al., 2008b), the immunocapture RT-PCR assay in the present study indicated that ORF3 protein is present on the surface of at least one-third of HEV virions in the culture supernatant of pJE03-1760F/wt-transfected cells, regardless of the absence of ORF3 protein on faecal HEV (Table 2). The pJE03-1760F/wt virions in the culture supernatant were captured by anti-ORF2 antibody at an increased efficiency of nearly 90% by prior treatment of the particles with bile (Table 2) or 3% Tween 20 (data not shown), and 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\(^{-1}\), which is markedly lower than that of HEV in a faecal suspension at 1.27–1.28 g ml\(^{-1}\). With regard to another non-enveloped hepatitis virus, hepatitis A virus (HAV), it has been reported that incomplete neutralization is observed for HAV released from cultured cells due to lipid-associated virions, which banded at a lower density in CsCl than normal, neutralizable virus (Lemon & Binn, 1985). Therefore, it is tempting to speculate that HEV may be released from infected cells as a lipid-associated virion, accompanied by ORF3 protein, and that the ORF3 protein is then dissociated from the virion after shedding in bile.

### Table 2. Results of the immunocapture RT-PCR assay

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Input (copies per well)</th>
<th>Captured HEV per input (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mAb H6225 (anti-HEV ORF2)</td>
</tr>
<tr>
<td>Without pre-treatment with bile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJE03-1760F/wt (48 days p.t.)</td>
<td>26 000</td>
<td>25.4</td>
</tr>
<tr>
<td>pJE03-1760F/wt (54 days p.t.)</td>
<td>115 000</td>
<td>29.3</td>
</tr>
<tr>
<td>pJE03-1760F/fs (48 days p.t.)</td>
<td>21 000</td>
<td>31.3</td>
</tr>
<tr>
<td>pJE03-1760F/fs (54 days p.t.)</td>
<td>165 000</td>
<td>29.8</td>
</tr>
<tr>
<td>ΔORF3 (48 days p.t.)</td>
<td>13 000</td>
<td>93.2</td>
</tr>
<tr>
<td>ΔORF3 (54 days p.t.)</td>
<td>26 000</td>
<td>94.9</td>
</tr>
<tr>
<td>JE03-1760F (faecal specimen)</td>
<td>8 900</td>
<td>96.6</td>
</tr>
<tr>
<td>With pre-treatment with bile‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJE03-1760F/wt (54 days p.t.)</td>
<td>3 300</td>
<td>79.3</td>
</tr>
<tr>
<td>pJE03-1760F/fs (54 days p.t.)</td>
<td>3 800</td>
<td>83.4</td>
</tr>
<tr>
<td>ΔORF3 (54 days p.t.)</td>
<td>300</td>
<td>92.3</td>
</tr>
<tr>
<td>JE03-1760F (faecal specimen)</td>
<td>1 000</td>
<td>95.6</td>
</tr>
</tbody>
</table>

*Viruses were derived from the culture supernatant of transfected cells (pJE03-1760F/wt, pJE03-1760F/fs or ΔORF3) at 48 or 54 days p.t. and from a faecal specimen containing the JE03-1760F strain.

†mAb against hepatitis B virus pre-S2 protein (Okamoto et al., 1985).
‡Prior to performing the immunocapture RT-PCR assay, 10 μl culture supernatant or faecal suspension was mixed with 100 μl bile and incubated at 37 °C for 2 h and then diluted 1:100 with PBS containing 0.1% BSA.
In support of our speculation, it has been reported previously 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 (Surjit et al., 2006). Tsg101 has been identified as the critical cellular protein required for budding of enveloped viruses, such as 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. Tsg101 normally plays a central role in the formation of multivesicular bodies (MVBs), where cellular vesicles bud away from the cytoplasm into the lumen to create the MVBs, and the MVB pathway is utilized in the budding of several enveloped viruses from the plasma membrane (Pornillos et al., 2002). Intriguingly, bluetongue virus (BTV; a non-enveloped virus in the family Reoviridae) can leave host cells as enveloped particles by budding from the plasma membrane, although the envelope is unstable and is rapidly lost (Mertens et al., 2005). BTV budding is mediated by the viral non-structural protein NS3, which contains the PSAP motif, by recruiting Tsg101 (Wirblich et al., 2006). Taken together, it is likely that the ORF3 protein promotes budding of 'enveloped' HEV particles by recruiting Tsg101.

In conclusion, using a recently established reverse genetics system for HEV utilizable in a robust cell-culture system, we have provided evidence that the ORF3 protein is essential for virion egress from infected cells, which had been predicted by Emerson et al. (2006) but not demonstrated thus far. In addition, the present study indicates that the ORF3 protein is present on the surface of HEV particles released from infected cells, and suggests that HEV particles released from infected cells are lipid-associated.

ACKNOWLEDGEMENTS

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; from the Ministry of Health, Labour and Welfare of Japan; and by a Jichi Medical University Young Investigator Award (to K.Y.).

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Hepatitis E virus with monoclonal antibody: correlation of pre-S

Hemagglutination assay of polypeptide coded by the pre-S region of


