Hepatitis E virus (HEV) causes acute and fulminant hepatitis E in humans. Genotype 1 and 2 HEVs have caused outbreaks of hepatitis E as water-borne epidemics, and genotype 3 and 4 HEVs were found in sporadic cases of hepatitis E, which were most likely zoonotic in origin (Okamoto, 2007). HEV is classified as the sole member of the genus Hepevirus (Emerson et al., 2004a) in the family Hepeviridae. 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 (ORFs), ORF1, ORF2 and ORF3 (Tam et al., 1991). The ORF1 encodes non-structural proteins including RNA helicase and RNA-dependent RNA polymerase (Agrawal et al., 2001; Koonin et al., 1992; Magden et al., 2001). ORF2 and ORF3 proteins are translated from a single subgenomic RNA (Graff et al., 2006; Huang et al., 2007). The ORF2 protein is the viral capsid protein, consisting of 660 aa. The ORF3 protein is a small protein (113–114 aa) that is essential for viral infectivity in animals (Graff et al., 2005; Huang et al., 2007). However, the replication mechanism and protein functions of HEV are not fully understood.

Propagation of HEV in vitro has been attempted in various cell lines (Divizia et al., 1999; Huang et al., 1992; Kazachkov et al., 1992; Meng et al., 1997; Wei et al., 2000), but an efficient cell culture system has not been developed. Several research groups have established infectious cDNA clones of HEV, which were observed to replicate in non-human primates or pigs (Emerson et al., 2001, 2004b; Graff et al., 2005; Huang et al., 2005, 2007; Panda et al., 2000). However, efficient HEV propagation in cultured cells has not yet been observed in any of these infectious cDNA clone systems, due to the inability of the virus progeny to spread to other cells in culture (Emerson et al., 2004b). Recently, we developed an efficient cell-culture system for HEV in PLC/PRF/5 and A549 cells using a genotype 3 HEV (strain JE03-1760F) that had been obtained from a faecal specimen from a Japanese hepatitis E patient (Lorenzo et al., 2008; Takahashi et al., 2007; Tanaka et al., 2007).

To develop a full-length infectious cDNA clone of HEV, RNA was extracted from the faecal specimen containing the JE03-1760F strain (Tanaka et al., 2007) using TRIzol LS (Invitrogen) and cDNA was synthesized using SuperScriptII (Invitrogen) with primers ENDr and 4927r (Supplementary Table S1, available in JGV Online). Using the synthesized cDNA as template, three fragments covering the entire JE03-1760F genome were amplified by PCR.

A full-length infectious cDNA clone (pJE03-1760F/wt) of a genotype 3 hepatitis E virus (HEV) (strain JE03-1760F) obtained from a faecal specimen was constructed in this study. Upon transfection of the capped in vitro transcripts of pJE03-1760F/wt into PLC/PRF/5 cells, the viral RNA levels in the culture supernatant started to increase on day 6 post-transfection (p.t.) and reached 10^7 copies ml^-1 on day 28 p.t. Detection of increasing numbers of cells with ORF2 protein expression by immunofluorescence assay at 5, 7, 11 and 15 days p.t. indicated the spread of HEV infection in cell culture. When the cDNA-derived virus in culture supernatant was inoculated into PLC/PRF/5 or A549 cells, it grew as efficiently as the faeces-derived virus in both cells, reaching 10^8 copies ml^-1 at 30 days post-inoculation. Our reverse genetics system for HEV that is usable in a robust cell-culture system will be useful for elucidation of the mechanism of HEV replication and functional roles of HEV proteins.

Construction of an infectious cDNA clone of hepatitis E virus strain JE03-1760F that can propagate efficiently in cultured cells

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with KOD plus ver. 2 (Toyobo) (Fig. 1). Fragment 1 (f1), which contains the T7 RNA polymerase promoter sequence upstream of the extreme 5’-end of the JE03-1760F genome, was amplified with primers f1-f and f1-r (Supplementary Table S1). The f2 fragment was amplified with primers f2-f and f2-r, and the f3 fragment with primers f3-f and f3-r; an AflII site was introduced as a genetic marker at the 3’ or 5’ end of the f2 and f3 fragments, respectively, by replacing T with C at nt 4784 and G with T at nt 4786, without amino acid substitution (Fig. 1). Fragment polyAT7φ, consisting of 31 nt of adenine and a T7 terminator sequence, was amplified by PCR with primers polyAT7φ-f and polyAT7φ-r using pTnT vector (Promega) as a template. An A overhang was added to the amplified blunt-ended fragments using A-Addition kit (Qiagen) and these were cloned into pT7 Blue T vector (Novagen). After confirming the sequence of the cloned fragments, the four fragments were ligated stepwise at NotI, AflII and SapI sites, and inserted into the HindIII–BamHI site of the pUC19AattII/SapI vector (kindly provided by Dr N. Ito, Gifu University, Japan). The full-length cDNA of the JE03-1760F strain that was constructed was designated pJE03-1760F/wt. In addition, a recombinant plasmid was constructed as a negative control. To achieve this, pJE03-1760F/wt plasmid DNA was digested with AatII from pJE03-1760F/wt by digestion with AatII followed by blunting and self-ligation.

First, we tried to use the nonlinearized genome plasmid as a template for in vitro transcription, but transcription was not completely terminated by the T7 terminator sequence. Therefore, using the NheI-linearized plasmid as a template, genomic RNA was transcribed with AmpliScribe T7-Flash Transcription kit (Epicentre Biotechnologies). The transcribed RNA was capped using ScriptCap m7G Capping System (Epicentre Biotechnologies) and purified. An aliquot (3 μg) of the capped RNA was transfected into subconfluent (60–80 % confluent) PLC/PRF/5 cells (ATCC no. CRL-8024), which were grown in culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10 % (v/v) fetal calf serum (FCS), 100 U penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 2.5 μg amphotericin B ml⁻¹ (Tanaka et al., 2007), in a well of six-well plates (Iwaki) in duplicate, using the TransIT-mRNA Transfection kit (Mirus Bio); the transfected cells were 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. Then, 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, 100 U penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 2.5 μg amphotericin B ml⁻¹. The collected medium was centrifuged at 800 g at 4 °C for 5 min and the supernatant was stored at −80 °C until use.

To monitor virus production in transfected cells (two wells each), HEV RNA levels in the culture supernatants of the transfected cells were serially quantified by real-time RT-PCR using the QuantiTect Probe RT-PCR kit (Qiagen) in a LightCycler apparatus (Roche Diagnostics) as described previously (Takahashi et al., 2008a). The HEV RNA titre decreased to approximately 10⁶ copies ml⁻¹ in all samples tested at 4 days p.t., due to the medium change on day 2 p.t. (Fig. 2a). Thereafter, a gradual decrease in HEV load was observed in the culture medium of the ΔORF1 mutant RNA-transfected cells, whose titre probably reflects residual amounts of the introduced RNA transcripts in the culture medium. On the other hand, in the pJE03-1760F/wt RNA-transfected cells, the viral RNA levels started to increase at 6 days p.t. and reached greater than 10⁷ copies ml⁻¹ at 28 days p.t. The viral RNA load in the medium was maintained in the order of 10⁷ copies ml⁻¹ in the pJE03-1760F/wt RNA transfection through to 60 days p.t.

An immunofluorescence assay was performed as described previously (Takahashi et al., 2008b). Briefly, on days 5, 7, 11 and 15 p.t., pJE03-1760F/wt RNA-transfected PLC/PRF/5 cells were fixed with 4 % paraformaldehyde, permeabilized with 0.2 % Triton X-100, incubated with a mouse monoclonal antibody (mAb) against the ORF2 protein (H6225) (Takahashi et al., 2008a) and stained with Alexa...
Fluor 488-conjugated anti-mouse IgG (Invitrogen). ORF2 protein expression was detectable in the transfected cells at 5 days p.t., and increasing levels of ORF2 antigens on days 7, 11 and 15 p.t. suggested the spread of HEV infection in cell culture (Fig. 2b).

For detection of ORF2 protein in the culture supernatant, Western blot analysis was performed using the 60 days p.t. samples. The culture supernatant of HEV RNA-transfected cells was directly mixed with the same amount of 2× SDS-PAGE sample buffer [125 mM Tris/HCl (pH 6.8), 4% SDS, 10% (w/v) sucrose, 10% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue]. Proteins in the samples were separated by SDS-PAGE on an 8% polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane (0.45 μm; Millipore). The membrane was incubated with anti-HEV ORF2 mAb (H6210) conjugated with horseradish peroxidase (HRP) (Takahashi et al., 2008a) and visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).
As shown in Fig. 2(c), a single 83 kDa band was detected in the culture medium of the pJE03-1760F/wt RNA transfection (lane 1) but not in that of the ΔORF1 mutant RNA transfection (lane 2). In addition, cell lysates prepared after DNA or RNA transfection were subjected to detection of ORF2 protein by Western blotting with H6210 mAb and enhanced chemiluminescence HRP-conjugated anti-mouse IgG from sheep (GE Healthcare). For preparation of cell lysates, DNA- or RNA-transfected cells on six-well plates were lysed in 100 μl lysis buffer [50 mM Tris/HCl (pH 8.0), 1% NP-40 and 150 mM NaCl] and the lysate was mixed with the same amount of 2× SDS-PAGE sample buffer. 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) or mock-transfected cells (lane 4) (Fig. 2c). For comparison, an expression plasmid, pCI-HEVORF2, for the ORF2 protein of strain JE03-1760F was constructed in the following way. The full-length ORF2 sequence of the JE03-1760F genome was amplified with primers ORF2-f and ORF2-r (Supplementary Table S1). The amplified DNA was cloned into the NheI–XbaI site of pCI vector (Promega), and the resulting plasmid, pCI-HEVORF2, was transfected into subconfluent PLC/PRF/5 cells in wells of a six-well plate using TransIT-LT1 Transfection reagent (Mirus Bio). The transfected cells were incubated at 37°C for 3 days and then analysed by Western blotting. Interestingly, the molecular mass of the ORF2 protein expressed by the pCI expression plasmid (Fig. 2c, lane 3) was slightly less than that detected in the pJE03-1760F/wt RNA-transfected cells.

The genomic region containing the AflII site (Fig. 1) was amplified by RT-PCR using primer 5811r for cDNA synthesis and primers 4511f and 5522r for PCR (Supplementary Table S1). In contrast with the original JE03-1760F strain in faeces, the amplified fragment from the culture supernatant of pJE03-1760F/wt RNA transfection was digestable with AflII (Fig. 2d), confirming that the propagated cDNA-derived virus (pJE03-1760F/wt) possessed the introduced genetic marker.

The pJE03-1760F/wt and original JE03-1760F strains were inoculated into PLC/PRF/5 or A549 cells (ATCC no. CCL-185), which were grown in maintenance medium on six-well plates at 1.0×10^3 or 1.0×10^4 copies per well, respectively. Virus inoculation and maintenance of inoculated cells were carried out as described previously (Tanaka et al., 2007). Fig. 3 indicates that the cDNA-derived pJE03-1760F/wt grew as efficiently as the original faeces-derived virus in both PLC/PRF/5 and A549 cells, reaching 10^6 or 10^5 copies ml^-1 at 30 days post-inoculation, respectively.

In the present study, an infectious cDNA clone of HEV with efficient propagation capability was successfully established using strain JE03-1760F. We previously established an infectious cDNA clone of HEV with efficient propagation capability was successfully established using strain JE03-1760F.
reported that the HEV RNA titre in the faecal suspension of strain JE03-1760F was markedly high, at 2.0 × 10^7 copies ml^{-1}, compared with the specimens from 10 other patients studied (less than 5.7 × 10^6 copies ml^{-1}) (Takahashi et al., 2007), suggesting that strain JE03-1760F has heightened replication activity. Therefore, the use of the JE03-1760F genome in the present study may have been the greatest factor contributing to the successful development of an infectious HEV cDNA clone with robust infection and propagation in vitro. The JE03-1760F genome has 29 unique nucleotide substitutions that were not seen in any of the 25 reported HEV isolates of the same genotype (Takahashi et al., 2007). One or some of these substitutions may be responsible for the high replication capability of HEV.

Interestingly, the ORF2 protein was detectable as a single band of 83 kDa following Western blot analysis of the supernatant and lysate of the pJE03-1760F/wt RNA-transfected cells, and the molecular mass was slightly greater than that of the protein transcribed by the pCI-HEVORF2 plasmid vector (Fig. 2c). In the reported transient overexpression systems, both glycosylated and non-glycosylated forms of ORF2 proteins have been detected. Jameel et al. (1996) reported that the ORF2 protein was detected in three molecular forms, including a 74 kDa non-glycosylated form and 82 and 88 kDa glycosylated forms. It was reported that the glycosylated form of ORF2 protein gradually shifted to the non-glycosylated form in the cytoplasm through the retro-translocation pathway (Surjit et al., 2007) and that only the non-glycosylated form was stable in the cytoplasm of mammalian cells (Torresi et al., 1999). However, the 83 kDa ORF2 protein detected in the culture supernatants and lysates of pJE03-1760F/wt RNA-transfected cells (Fig. 2c) and cells infected with cell culture-generated HEV (data not shown) is likely to be glycosylated. In support of our speculation that the glycosylated form of ORF2 protein has a role in the HEV replication cycle, 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. In addition, it was shown that the recombinant replicase domain of HEV ORF1 protein is localized on the endoplasmic reticulum membrane (Rehman et al., 2008). HEV particles in the culture supernatant banded at a low buoyant density of 1.15–1.16 g ml^{-1} in sucrose, suggesting that HEV virions released from infected cells associate with lipids (Takahashi et al., 2008b). Therefore, the ORF2 capsid protein existing as a glycoprotein may be advantageous for virion assembly if cellular membranes play an important role in HEV particle maturation.

In conclusion, we established a reverse genetics system for HEV that is utilisable in a robust cell-culture system. This system will be useful for further elucidation of the mechanism of HEV replication and the functional roles of HEV proteins.

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