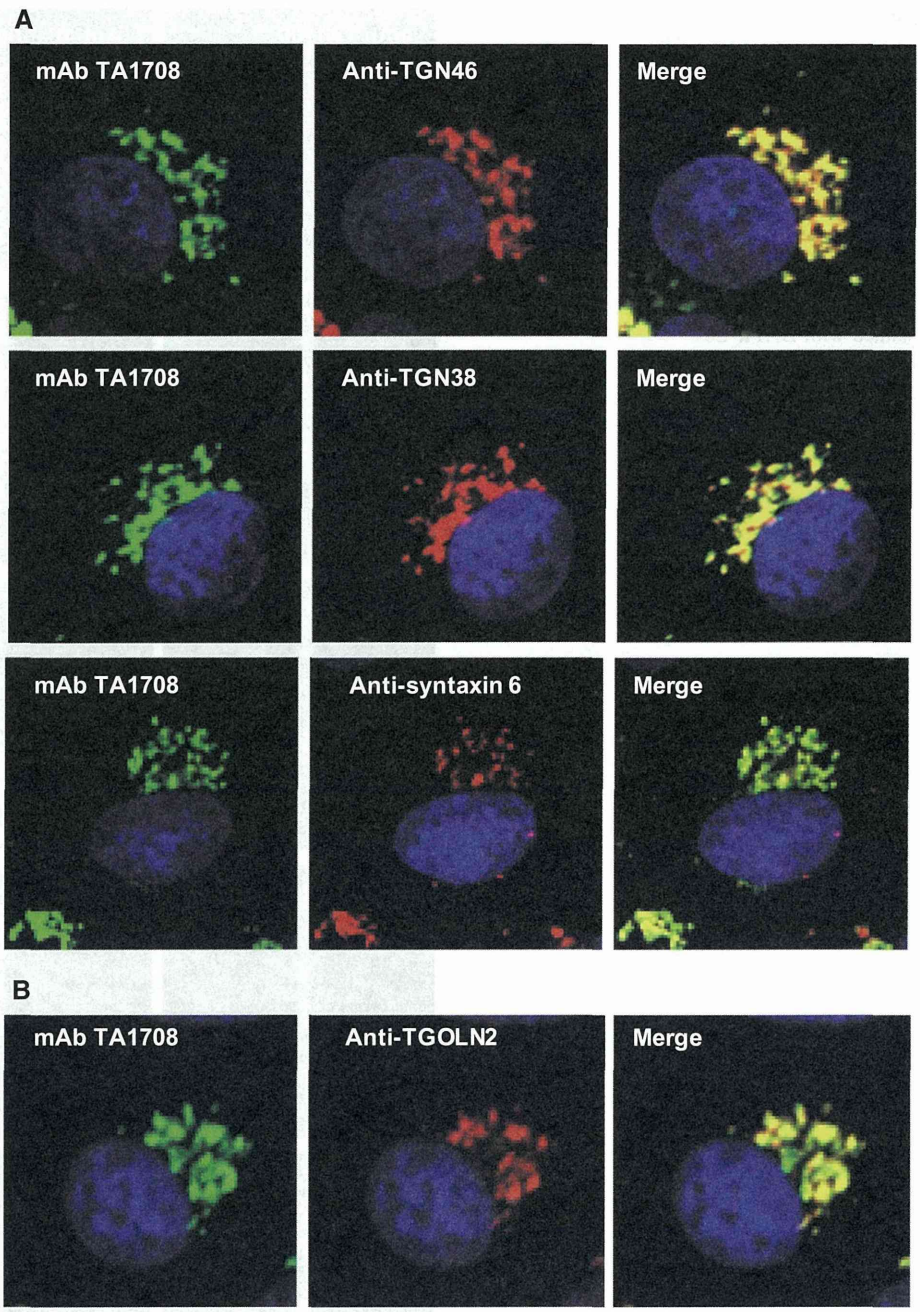

Fig. 4 Co-localization of the antigen recognized by mAb TA1708 with TGN markers. PLC/PRF/5 cells were fixed and double-stained with mAb TA1708 labeled with Alexa Fluor 488 and rabbit anti-TGN46, anti-TGN38, or anti-syntaxin 6 antibodies labeled with Alexa Fluor 594 (**A**) or rabbit anti-TGOLN2 antibodies labeled with Alexa Fluor 594 (**B**). The nuclei were stained with DAPI. Co-localization is indicated by yellow staining. All images are representative of two independent experiments

Intracellular localization of the expressed TGOLN2 recombinant protein and the antigen recognized by mAb TA1708

We subsequently examined the co-localization of Myc-tagged TGOLN2 protein and the antigen recognized by mAb TA1708 using immunofluorescence confocal microscopy. First, to confirm the specific detection of the Myc-tagged TGOLN2 expressed in the transfected cells, PLC/PRF/5 cells transfected with pFLAG-Myc-CMV-22-TGOLN2 or pFLAG-Myc-CMV-22 empty vector were stained with Alexa Fluor 488–conjugated anti-Myc mAb. The signal of Myc-tagged TGOLN2 stained by anti-Myc antibody was visible only in the cytoplasm, mainly on the nuclear periphery, of cells transfected with pFLAG-Myc-CMV-22-TGOLN2 (Fig. 5, middle-left panel), even though the specific signals of intracellular TGOLN2 were observed in the cytoplasm, primarily on the fringe and unevenly in the nucleus (Fig. 4B). In contrast, no specific signals were observed in the cells transfected with empty vector (Fig. 5, upper-left panel). These results indicate the specific detection of Myc-tagged TGOLN2 in the expressed cells by immunofluorescence assay using an anti-Myc antibody.

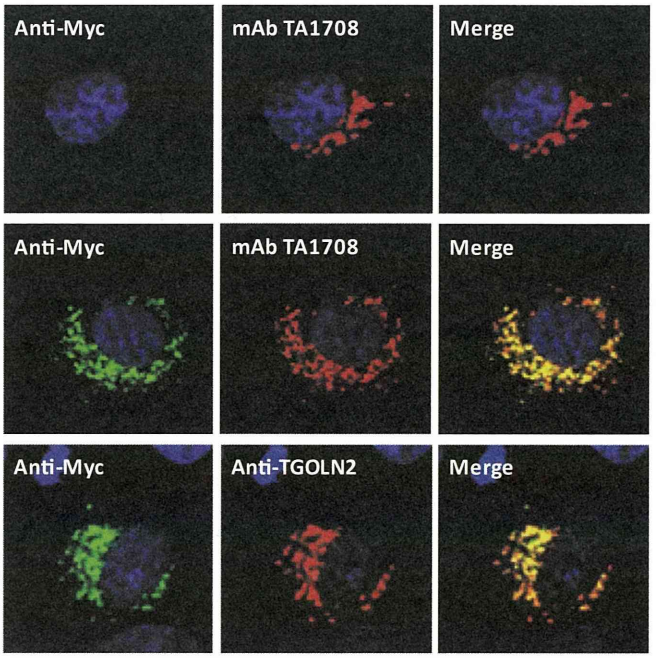

Fig. 5 Co-localization of the antigen recognized by mAb TA1708 with Myc-tagged TGOLN2 recombinant protein. PLC/PRF/5 cells were transfected with pFLAG-Myc-CMV-22 empty vector or pFLAG-Myc-CMV-22-TGOLN2. Cells were fixed and double-stained with anti-Myc mAb labeled with Alexa Fluor 488 and mAb TA1708 labeled with Alexa Fluor 568 or rabbit anti-TGOLN2 antibody labeled with Alexa Fluor 594. The nuclei were stained with DAPI. Co-localization is indicated by yellow staining. All images are representative of two independent experiments

To examine whether the antigen recognized by mAb TA1708 co-localizes with the expressed TGOLN2 protein, PLC/PRF/5 cells transfected with pFLAG-Myc-CMV-22-TGOLN2 were subjected to immunofluorescence assay. At least 20 different cells expressing Myc-tagged TGOLN2 were analysed in two independent experiments. A high degree of co-localization (97.5 ± 2.5 %) was observed in the cytoplasm (Fig. 5, middle panel). Similarly, co-localization was also demonstrated by double staining with anti-TGOLN2 and anti-Myc antibodies (Fig. 5, lower panel).

Membrane-associated HEV particles are generated intracellularly

In our previous study, an immunofluorescence assay using anti-ORF3 mAbs and antibodies against CD63, a MVB marker protein, revealed that ORF3 proteins are co-localized with CD63 in HEV-infected cells [31]. Furthermore, the mAb TA1708 against the membrane on the surface of HEV particles reacts with intracellular antigens (Fig. 2), thus suggesting that mature membrane-associated HEV particles are generated before being released from infected cells. To test our speculation that the membrane-associated virus particles are generated intracellularly, lysates of cells transfected with wild-type RNA were subjected to equilibrium centrifugation in a sucrose density gradient (Fig. 6). The viral particles in the cells transfected with the wild-type RNA exhibited a biphasic pattern, peaking at 1.15 and 1.26 g/ml, while the particles released into the culture supernatant banded in a single peak of 1.16 g/ml.

To characterize the cell-lysate-derived particles that were distributed into two major fractions, immunocapture RT-PCR was performed using TA1708, anti-ORF2 and anti-ORF3 mAbs, with or without prior treatment with 0.1 % sodium deoxycholate (Table 3). The higher-density particles (1.26 g/ml) in the cell lysates (fraction 3) of the cells transfected with wild-type RNA were efficiently captured by the anti-ORF2 mAb (81.5 %), but not by the TA1708 (0.0 %) or anti-ORF3 (0.8 %) mAbs, with or without prior treatment with 0.1 % sodium deoxycholate, similar to what was observed using the fecal supernatant (Table 1). In contrast, the lower-density particles (1.15 g/ml) derived from lysates of cells transfected with wild-type RNA (fraction 2) were captured by mAb TA1708 (46.0 %) without prior treatment with 0.1 % sodium deoxycholate as efficiently as the wild-type particles in the culture supernatant (fraction 1) (46.3 %). When the particles in fractions 1 and 2 were treated with 0.1 % sodium deoxycholate, the rate of capture by mAb TA1708 was reduced to 13.5 % and 13.9 %, respectively. On the other hand, the lower-density particles (fraction 2) were trapped by both anti-ORF2 and anti-ORF3 mAbs after treatment with 0.1 % sodium deoxycholate (64.3 % and 90.6 %, respectively), as efficiently as the wild-type particles in the culture supernatant (fraction 1) (62.2 % and 61.0 %, respectively). Furthermore, the viruses were not captured by anti-ORF2 or anti-ORF3 mAbs without treatment with 0.1 % sodium deoxycholate (11.6 % and 9.6 %, respectively), similar to the results obtained with the wild-type particles in the culture supernatant (9.5 % and 9.1 %, respectively) (Table 3). These results indicate that HEV particles with lipid membranes and ORF3 proteins on their surface are present abundantly in the lysates of cells transfected with wild-type HEV RNA.

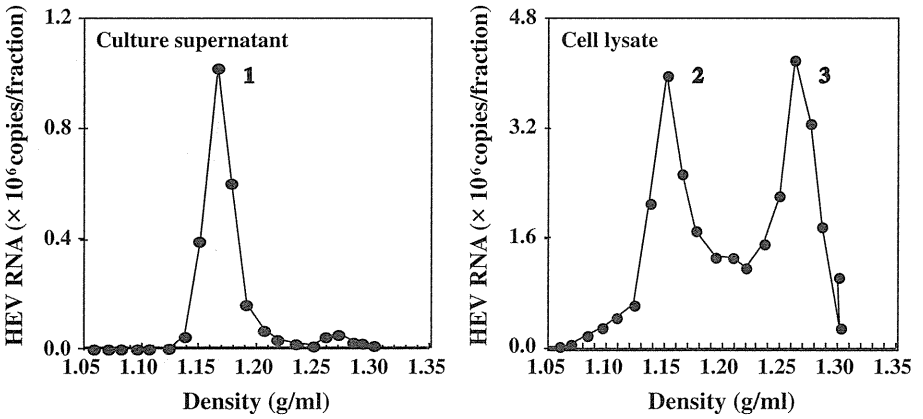

Fig. 6 Sucrose density gradient fractionation of the wild-type virus in the culture supernatants and cell lysates. The peak fractions (numbers 1 to 3) were subjected to immunocapture RT-PCR (see Table 3)

Table 3 Reactivity of TA1708, anti-ORF2 and anti-ORF3 mAbs with HEV particles in the culture supernatants and cell lysates with or without prior treatment with detergent, as evaluated using immunocapture RT-PCR

Virus[a]	% of captured HEV in the total HEV per well		
	mAb TA1708	mAb H6225 (anti-ORF2)	mAb TA0536 (anti-ORF3)
Without pre-treatment with detergent			
Culture supernatant			
Fraction 1 (1.16 g/ml)	46.3	9.5	9.1
Cell lysate			
Fraction 2 (1.15 g/ml)	46.0	11.6	9.6
Fraction 3 (1.26 g/ml)	0.7	64.5	2.0
With pre-treatment with detergent[b]			
Culture supernatant			
Fraction 1 (1.16 g/ml)	13.5	62.2	61.0
Cell lysate			
Fraction 2 (1.15 g/ml)	13.9	64.3	90.6
Fraction 3 (1.26 g/ml)	0.0	81.5	0.8

[a] Viruses were derived from the culture supernatant or lysate of pJE03-1760F/wt-transfected cells at 28 days post-transfection, and the peak fractions, with the indicated fraction number and sucrose density in parentheses (see Fig. 3), were subjected to immunocapture RT-PCR

[b] Prior to performing the immunocapture RT-PCR assay, 6 µl of the sucrose fraction was mixed with 60 µl of 0.11 % sodium deoxycholate, incubated at 37 °C for 2 hours, and then diluted 1:10 with PBS containing 0.1 % BSA

Discussion

Our previous study showed that an intact PSAP motif in the ORF3 protein is required for the formation and release of membrane-associated HEV particles possessing ORF3 proteins on the surface [30]. Moreover, we demonstrated that HEV recruits Tsg101 via its PSAP motif in the ORF3 protein and requires the L-domain function for virion release from infected cells, and also that the enzymatic activity of Vps4 is involved in virus release [31]. These results suggest that, although HEV is known to be a non-enveloped virus, it requires the MVB pathway for its release from infected cells. However, the origin of the membrane has not yet been clarified. In the present study, we generated and utilized a murine mAb against membrane-associated HEV particles, designated TA1708, and found that the mAb TA1708 binds specifically to a component of the membrane on the surface of HEV particles in an immunocapture RT-PCR assay (Table 1). In addition, digitonin treatment revealed that the membrane on the surface of viral particles shed into the culture supernatant is a lipid membrane (Fig. 1 and Table 2).

It remains unknown whether membrane-associated HEV particles are produced in the cytoplasm or at the cell surface [2]. Many enveloped viruses are known to complete their replication cycle by budding from the plasma membrane [10, 16, 18]. HIV, Ebola virus and other RNA viruses utilize the cellular ESCRT machinery to promote their escape from host cells by redirecting ESCRT complexes to the cell surface, where they appear to drive the budding and fission of the viral particles [10, 14, 27]. In our previous study, an immunofluorescence analysis revealed that the ORF3 protein is co-localized with CD63 and Tsg101 in the cytoplasm of HEV-infected cells [30, 31], thus suggesting that mature membrane-associated HEV particles are generated before their release from the surface of infected cells. In this study, we analyzed the subcellular localization of the antigen recognized by mAb TA1708 using immunofluorescence confocal microscopy. Specific signals were observed only in the cytoplasm, not on the plasma membrane (Fig. 2). Furthermore, the antigens were co-localized with TGOLN2, which has been established to be a TGN marker (Fig. 4). HEV particles with lipid membranes and the ORF3 protein on their surface were found abundantly in the lysates of cells infected with wild-type HEV (Fig. 6 and Table 3). Taken together, these findings indicate that HEV forms membrane-associated particles in the cytoplasm, likely by utilizing the cellular ESCRT machinery in the cytoplasm, not at the cell surface.

Lai et al. [22] reported that HCV egress requires the motility of early to late endosomes, which is microtubule-dependent. Additionally, they postulated that following the assembly of virus particles in juxtaposition to lipid droplets, the HCV particles are transported through early to late endosomes to the plasma membrane, where the membrane of late endosomes is fused with the plasma membrane to release virions into the extracellular milieu. Recently, Tamai et al. [41] reported that the Hrs-dependent exosomal pathway plays an important role in HCV release. In most herpesviruses, the final envelopment occurs at the Golgi, post-Golgi compartments, such as the TGN, or endosomes [8, 13]. Mori et al. [29] found that human herpesvirus 6 (HHV-6) buds at TGN-associated membranes, which contain CD63 and TGN46, and incorporates CD63 into virions. In addition, the virions are released together with internal vesicles (exosomes) through MVBs via the cellular exosomal pathway [29]. In the present study, we found, using double immunofluorescence staining, that mAb TA1708, which was raised against membrane-associated HEV particles, specifically recognizes TGOLN2, an intracellular antigen derived from the TGN, (Figs. 4 and 5). These results suggest that TGOLN2 derived from the TGN is a surface antigen of membrane-associated HEV particles. Contrary to our expectation, TGOLN2 recombinant protein was not detectable by western blot analysis, even under non-reducing or non-denaturing conditions, or immunoprecipitation using mAb TA1708 (data not shown), suggesting that the epitope of TGOLN2 that is present on the surface of virus particles, recognizable by mAb TA1708, is conformational and likely to be different from those expressed on the TGOLN2 recombinant protein. However, in an immunofluorescence assay, the Myc-tagged TGOLN2 recombinant protein was detectable by mAb TA1708, probably due to the maintenance of the three-dimensional structure of the recombinant protein expressed in cells. The co-localization of the antigen recognized by mAb TA1708 with the Myc-tagged TGOLN2 recombinant protein demonstrated by immunofluorescence assay using TA1708 and anti-Myc mAbs (Fig. 5) supports our notion that mAb TA1708 recognizes TGOLN2.

TGOLN2 is a protein encoded by the TGOLN2 gene in humans [34]. It has alternative names, including TGN38, TGN46, TGN48, TGN51 and TTGN2 [20, 25, 34], reflecting the presence of several isoforms of TGOLN2, which are produced by alternative splicing. This gene encodes a type I integral membrane protein that is localized to the TGN, a major sorting station for secretory and membrane proteins [34]. The encoded protein cycles between the TGN and early endosomes and may play a role in exocytic vesicle formation. Syntaxin 6 protein is also located at the TGN and mediates TGN vesicle trafficking events, likely via transport from the TGN to the endosome [4]. However, the present immunofluorescence studies (Fig. 4) suggested that mAb TA1708 is an antibody against a TGN protein that is recognizable by antibodies raised against TGN38, TGN46 and TGOLN2 rather than syntaxin 6.

Our previous study showed that the MVB pathway is important for the release of HEV particles and that ORF3 proteins are co-localized with CD63 in the cytoplasm [31]. In the present study, it was found that mAb TA1708 reacts with the intracellular antigen TGOLN2, according to the findings of double staining immunofluorescence analysis (Fig. 4). These results suggest that ORF3 proteins are transported on to the membrane of MVB by the early endosome via the vesicular transport system of TGN. It is likely that endosomal transport is involved in the release of HEV particles.

In conclusion, the present study revealed that membrane-associated HEV particles are present abundantly in the lysates of infected cells. In addition, double immunofluorescence staining showed that membrane-associated HEV particles retain the antigenicity of TGOLN2 derived from the TGN on the surface of the particles. These results indicate that the membrane of membrane-associated HEV particles is derived from the intracellular membrane, not from the cell surface. Further studies are required to elucidate whether the lipid membrane on the surface of HEV particles is derived not only from the TGN, but also from the Golgi apparatus or endosomes and whether mature virions are released via the exosomal secretion pathway, similar to what has been observed for other enveloped viruses, such as HCV and HHV-6 [22, 29, 41].

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Rat hepatitis E virus derived from wild rats (*Rattus rattus*) propagates efficiently in human hepatoma cell lines☆

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ABSTRACT

Although rat hepatitis E virus (HEV) has been identified in wild rats, no cell culture systems for this virus have been established. A recent report suggesting the presence of antibodies against rat HEV in human sera encouraged us to cultivate rat HEV in human cells. When liver homogenates obtained from wild rats (*Rattus rattus*) in Indonesia were inoculated onto human hepatocarcinoma cells, the rat HEV replicated efficiently in PLC/PRF/5, HuH-7 and HepG2 cells, irrespective of its genetic group (G1–G3). The rat HEV particles released from cultured cells harbored lipid-associated membranes on their surface that were depleted by treatment with detergent and protease, with the buoyant density in sucrose shifting from 1.15–1.16 g/ml to 1.27–1.28 g/ml. A Northern blotting analysis revealed genomic RNA of 7.0 kb and subgenomic RNA of 2.0 kb in the infected cells. The subgenomic RNA of G1–G3 each possessed the extreme 5′-end sequence of GUAGC (nt 4933–4937), downstream of the highly conserved sequence of GAAUAACA (nt 4916–4923). The establishment of culture systems for rat HEV would allow for extended studies of the mechanisms of viral replication and functional roles of HEV proteins. Further investigation is required to clarify the zoonotic potential of rat HEV.

1. Introduction

Hepatitis E virus (HEV) is the causative agent of acute and fulminant hepatitis in many developing countries in Asia and Africa, where the poor sanitary conditions are associated with fecal–oral transmission (Emerson and Purcell, 2013). However, hepatitis E has been found to be endemic in industrialized countries, including Japan, the United States and Europe, where the zoonotic food-borne transmission of HEV from domestic pigs, wild boars and wild deer to humans plays an important role (Colson et al., 2010; Meng, 2013; Takahashi and Okamoto, 2013; Tei et al., 2003; Yazaki et al., 2003). Hepatitis E is typically a self-limiting disease with variable severity, presenting as acute icteric hepatitis with clinical symptoms. However, chronic HEV infection has recently been documented in immunocompromised patients, such as solid-organ transplant recipients and human immunodeficiency virus-infected patients (Dalton et al., 2009; Gerolami et al., 2008; Haagsma et al., 2009; Kamar et al., 2013; Zhou et al., 2013). HEV is classified as the sole member of the genus *Hepevirus* of the family *Hepeviridae* (Meng et al., 2012). Its genome is a single strand, positive-sense RNA of approximately 7.2 kilobases (kb) in size and contains a short 5′-untranslated region (5′-UTR), three open reading frames (ORFs: ORF1, ORF2 and ORF3) and a short 3′-UTR terminated by a poly(A) tract (Tam et al., 1991). ORF1 at the 5′ end of the genome encodes several non-structural proteins involved in replication, while ORF2 codes for a capsid protein of 660 amino acids (aa). ORF3 encodes a small protein of only 113–114 aa that is required for viral infectivity in animals (Graff et al., 2005; Huang et al., 2007) and virion egress (Emerson et al., 2010; Yamada et al., 2009a). ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA measuring 2.2 kb in length (Graff et al., 2006; Ichiyama et al., 2009).

☆ The nucleotide sequences of rat HEV isolates reported herein have been assigned DDBJ/EMBL/GenBank accession nos. AB897758–AB897782.

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Four genotypes (genotypes 1–4) of HEV have been identified in humans (Okamoto, 2007). Genotype 1 and 2 HEVs are restricted to humans and often associated with large outbreaks and epidemics in developing countries in Asia and Africa. Genotype 3 and 4 HEVs have a wider host range with several animal species as hosts, including humans as well as pigs, wild boars, deer, mongooses and rabbits (Meng et al., 1997; Nakamura et al., 2006; Sonoda et al., 2004; Takahashi et al., 2004; Zhao et al., 2009): the molecular epidemiological evidence on transmission from reservoirs to humans indicates that genotype 3 and 4 HEVs are zoonotic (Colson et al., 2010; Li et al., 2005; Tei et al., 2003; Yazaki et al., 2003). Recently, additional new putative genotypes of HEV have been identified in wild boars in Japan (Takahashi et al., 2010a, 2011), ferrets in the Netherlands (Raj et al., 2012) and wild rats (*Rattus norvegicus*) in Germany (Johne et al., 2010). Rat HEV strains have been identified in other countries including the United States, Vietnam, Indonesia and China (Li et al., 2011, 2013b; Mulyanto et al., 2013; Purcell et al., 2011). The phylogenetic analysis revealed that rat HEV strains are segregated into three distinct genetic groups [a German type (G1), Vietnamese type (G2) and Indonesian type (G3)] that differ from each other by 19.5–23.5 (22.0 ± 1.7)% over the entire genome (Mulyanto et al., 2014). Of interest, various rat HEV strains classifiable into three genetic groups have been identified in Indonesia, suggesting the global distribution of heterogeneous rat HEV strains in this country (Mulyanto et al., 2014). However, whether rat HEV can be transmitted to humans remains unclear, and no cell culture system for rat HEV has yet been established (Johne et al., 2010).

Recently, Dremsek et al. (2012) reported the detection of anti-rat HEV antibodies in human serum samples, which encouraged us to cultivate rat HEV in human cell lines. In the present study, homogenates of liver specimens obtained from wild rats (*Rattus rattus*) with high viral load in Lombok and Java, Indonesia (Mulyanto et al., 2014), were inoculated onto human hepatocarcinoma cells in order to propagate rat HEV. The rat HEV replicated efficiently in cultured human cells, and the progeny viruses in the culture supernatant and genomic RNAs in the cell lysates were characterized to support the successful propagation of rat HEV in human cells.

2. Materials and methods

2.1. Liver specimens from wild rats with high viral load

In our previous study, paired serum and liver specimens were obtained from 136 black wild rats (*R. rattus*) in Solo (also called Surakarta), a city in Central Java, between September 24 and October 5, 2012 and from 233 wild rats on Lombok Island between October 2 and 22, 2012, and the serum samples were used to investigate the prevalence of rat HEV infection and analyze rat HEV genomes (Mulyanto et al., 2014). In the present study, the rat HEV RNA in the liver specimens of 99 viremic rats was quantitated, and liver homogenate samples with a high load of rat HEV belonging to each of three genetic groups (G1, G2 or G3) were used as inocula in cell cultures, as described below (see Table 1). All paired serum and liver samples were stored at −20 °C in Indonesia and −80 °C after being sent to Japan and preserved until testing.

2.2. Qualitative and quantitative detection of rat HEV RNA

Total RNA was extracted from 20 to 100 μl of each liver homogenate or cell lysate using the TRIzol reagent (Life Technologies, Carlsbad, CA) or 10–100 μl of each culture medium using the TRIzol LS reagent (Life Technologies) according to the manufacturer's instructions. In order to detect rat HEV RNA in the liver homogenate or culture medium, a portion of the ORF1 and ORF2 junction region (primarily the ORF1 region) was amplified *via* nested reverse transcription (RT)-PCR using the primer sets HE607 (sense) and HE604 (antisense) in the first round and HE608 (sense) and HE606 (antisense) in the second round (ORF1-PCR), as previously described (Mulyanto et al., 2013). These sets generated amplification products of 899 base pairs (bp) (nt 4098–4996) and 880 bp (nt 4103–4982), respectively; the nucleotides are numbered in accordance with the prototype rat HEV strain [rat/R63/DEU/2009 (GU345042)], unless otherwise stated. The RT-PCR assay was performed in duplicate, and its reproducibility was confirmed. The specificity of the RT-PCR assay was verified using a sequence analysis, as described below.

The RNA of rat HEV was quantitated in various specimens using real-time RT-PCR according to the previously described method (Mulyanto et al., 2014), employing an *in vitro*-transcribed rat HEV RNA as a standard. Briefly, using the pT7Blue T-Vector (Merck Millipore, Tokyo, Japan) containing a 341-nt fragment of ratIDE079 cDNA (nt 35–375) as a template and the AmpliScribe™ T7-*Flash*™ Transcription Kit (Epicentre Biotechnologies, Madison, WI), rat HEV RNA was transcribed *in vitro*, purified and used as a standard of rat HEV RNA. The RNA preparations obtained from the liver homogenates, culture media, cell lysates and fractions *via* ultracentrifugation (see below) were subjected to real-time RT-PCR with a QuantiTect Probe RT-PCR Kit (QIAGEN, Tokyo, Japan) using the sense primer HE655 [5′-CCA CGG GGG TTA ATA CTG C-3′ (nt 36–54)], the antisense primer HE656 [5′-CGG ATG CGA CCA AGA AAC AG-3′ (nt 189–208)] and a probe (HE657-P) consisting of an oligonucleotide with a 5′-reporter dye (6-carboxyfluorescein, FAM) and a 3′-quencher dye (6-carboxytetramethyl-rhodamine, TAMRA) [5′-FAM-CGG CTA CCG CCT TTG CTA ATG C-TAMRA-3′ (nt 81–102)] on an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies). The thermal cycler conditions were 50 °C for 30 min, 95 °C for 15 min and 50 cycles of 94 °C for 15 s followed by 56 °C for 30 s. From standard dilutions analysis, the sensitivity of this real-time RT-PCR assay was estimated to be 2–5 copies/test or 20–50 copies/ml (when 100 μl of samples were used). It is expected that the sensitivity of this assay is the same for the genetic groups 1, 2 and 3, since the primers and a probe used were derived from well-conserved genomic area among the three genetic groups. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate, and the mean value was adopted.

2.3. Sequence analysis of the PCR products

The amplification products were sequenced directly on both strands using a BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies) on an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies). The sequence analysis was performed using the Genetyx software program (version 11.1.2; Genetyx Corp., Tokyo, Japan), and multiple alignments were generated by the CLUSTAL Omega software program (version 1.2.0) (Goujon et al., 2010). Phylogenetic trees were constructed according to the neighbor-joining method (Saitou and Nei, 1987) with the Kimura two-parameter model and 1000 replicates of bootstrap resampling, as implemented in the MEGA5 software program (version 5.2.1) (Tamura et al., 2011).

2.4. Preparation of inocula for cell culture

A piece of liver tissue (200 mg) was minced with a razor blade and homogenized with a BioMasher II (Nippi Incorporated, Tokyo, Japan) in the presence of 1.8 ml of phosphate buffered saline (pH 7.5) without Mg^{2+} and Ca^{2+} [PBS(−)] and clarified *via* centrifugation in a high-speed refrigerated microcentrifuge (Tomy Seiko, Tokyo, Japan) at 7900 × *g* at 4 °C for 10 min, and a clear supernatant was obtained. The 10% (w/v) homogenates were used as the inoculum. Aliquots of culture supernatant containing the rat HEV progeny

Table 1
Paired serum and liver specimens obtained from wild rats infected with rat HEV.

Sample No.	Weight (g)	Sex	Serum samples[a]					Liver samples	
			Rat anti-HEV IgG (OD)[b]	Rat HEV RNA				Isolate name of rat HEV	Rat HEV RNA in 10% (w/v) liver homogenate (copies/ml)
				Copies/ml	Isolate name	Accession No.	Genetic group		
SL006	110	F	0.044 (−)	3.0×10^2	ratESOLO-006S	AB847310	G1	ratESOLO-006L	2.5×10^6
095	48	F	0.048 (−)	1.2×10^5	ratELOMB-095S	AB847363	G2	ratELOMB-095L	8.3×10^7
131	158	M	2.218 (+)	1.7×10^5	ratELOMB-131S	AB847373	G2	ratELOMB-131L	8.4×10^7
159	76	F	2.005 (+)	1.5×10^5	ratELOMB-159S	AB847381	G2	ratELOMB-159L	9.6×10^7
213	129	F	>3.000 (+)	1.1×10^5	ratELOMB-213S	AB847399	G2	ratELOMB-213L	4.8×10^8
SL010	100	F	2.762 (+)	5.0×10^4	ratESOLO-010S	AB847312	G3	ratESOLO-010L	1.1×10^8
SL045	100	M	>3.000 (+)	9.7×10^3	ratESOLO-045S	AB847323	G3	ratESOLO-045L	5.1×10^6
SL061	80	F	2.700 (+)	5.5×10^3	ratESOLO-061S	AB847327	G3	ratESOLO-061L	1.2×10^6

[a] Data were retrieved from our previous report (Mulyanto et al., 2014).
[b] Samples with OD values equal to or greater than the cutoff value (0.234) were considered to be positive for rat anti-HEV IgG (Mulyanto et al., 2014).

were also used as the inoculum for supernatant passage following dilution with PBS(−) containing 0.2% (w/v) bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO). Prior to inoculation with the liver homogenates and culture supernatant, the virus stocks were subjected to purification by passaging them through microfilters with a pore size of 0.45 and 0.22 μm (Millex-GV; Millipore Corp., Bedford, MA).

2.5. Cell culture and virus inoculation

Three human hepatocarcinoma cell lines, including PLC/PRF/5 (ATCC No. CRL-8024; American Type Culture Collection, Manassas, VA), HuH-7 (No. JCRB0403; RIKEN BRC Cell Bank, Tsukuba, Japan) and HepG2 (No. RCB0459; RIKEN BRC Cell Bank), as well as a human lung cancer cell line, A549 (No. RCB0098; RIKEN BRC Cell Bank), were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO Cat. No.12800-058, Life Technologies), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HANA-NESCO BIO, Tokyo, Japan), 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 2.5 μg/ml of amphotericin B (medium A) at 37 °C in a humidified 5% CO_2 atmosphere, as previously described (Jirintai et al., 2012; Tanaka et al., 2007). For virus inoculation, cells (3×10^5 cells/ml) in 2.0 ml of medium were added to each well (diameter of 3.5 cm) of a six-well microplate (IWAKI, Tsukuba, Japan) three days before virus infection. Monolayers of cultured cells in the six-well microplates were washed three times with 1 ml of PBS(−) and 0.2 ml of homogenized liver suspension that had been prepared as described above, or culture supernatant was inoculated into each well. One hour after inoculation at room temperature in wells with PLC/PRF/5 or A549 cells (or additional overnight cultivation at 35.5 °C in wells with HuH-7 or HepG2 cells following the addition of 0.8 ml of medium A), the solution was removed and then the wells were washed five times with 1 ml of PBS(−), and then 2 ml of medium A was added to the wells with PLC/PRF/5 cells, medium A supplemented with 1.0% (v/v) dimethyl sulfoxide (DMSO) was added to the wells with HuH-7 or HepG2 cells and medium B was added to the wells with A549 cells. The medium B used for virus propagating in the A549 cells consisted of 50% DMEM and 50% medium 199 (GIBCO Cat. No. 31100-027, Life Technologies) containing 2% (v/v) heat-inactivated FBS and 30 mM $MgCl_2$ (final concentrations); the other supplements were the same as those used in medium A. The propagation was performed at 35.5 °C in a humidified 5% CO_2 atmosphere. Next, every other day, one-half (1 ml) of the culture medium was replaced with fresh medium, and the collected media were stored at −80 °C until virus titration. In this study, triplicate sets of virus specimens were inoculated in parallel into the cultivated cells in a six-well plate. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells, as described above. The infected tissue culture microplates were examined daily under an inverted microscope for potential cytopathic effects (CPE), using microplates inoculated with a liver homogenate from a rat HEV-negative rat as the negative control.

2.6. Equilibrium centrifugation in sucrose density gradient

Equilibrium centrifugation was performed in a sucrose density gradient, as previously described (Takahashi et al., 2010b) with slight modifications. The density gradient was prepared in an SW60Ti tube (Beckman Coulter, Inc., Brea, CA) [0.5 ml each of 65% and 60% (w/w) sucrose, 0.6 ml each of 50%, 40%, 30%, and 20% (w/w) sucrose and 0.4 ml of 10% (w/w) sucrose in Tris–HCl buffer (0.01 M, pH 7.5) supplemented with 1 mM EDTA and 150 mM NaCl (TEN)]. One hundred microliters each of serum sample positive for rat HEV, 10% (w/v) liver homogenate containing rat HEV and culture supernatant containing cell culture-generated rat HEV as well as that treated with 0.5% (w/v) sodium deoxycholate and 0.5% (w/v) trypsin at 37 °C for 2 h were layered onto the surface of the gradient. The tube was centrifuged at 179,200 × *g* at 10 °C for 48 h, and 150-μl fractions were then recovered from the surface. The density of each fraction was measured using refractometry, and the titer of rat HEV RNA in each fraction was determined according to real-time RT-PCR, as described above.

2.7. Preparation of a digoxigenin (DIG)-UTP-labeled negative-strand probe for rat HEV RNA

In order to prepare an RNA probe for the Northern blotting analysis of rat HEV RNA, the RNA extracted from a serum sample containing the ratELOMB-131S strain (AB847373) belonging to genetic group G2 was reverse transcribed using SuperScript II Reverse Transcriptase (Life Technologies) with a primer HE691 [5′-YCC CAT CCC ACC ADA CCC A-3′ (Y = T or C and D = A, T or G); nt 6623–6642]. PCR [(645 bp, including rat HEV sequence of 607 bp (nt 5532–6138)] was subsequently performed with primers derived from the ORF2 region containing the restriction enzyme sites of *Sal*I and *Bam*HI in the forward [RatHEVSal_F5532 (5′-TTT TGT CGA CCG GTA TCA ATG TCG TTC TGG-3′; the *Sal*I site is underlined), nt 5532–5551] and reverse [RatHEVBam-T7_R6119 (5′-TTT TGG ATC CTA ATA CGA CTC ACT ATA GGC TCT CCA TTC TCG GAC AC-3′; the *Bam*HI site is underlined and the T7 transcription promoter sequence is double underlined); nt 6119–6138] primers, respectively. In order to synthesize the negative-sense strand of rat HEV RNA, a T7 transcription promoter sequence was also

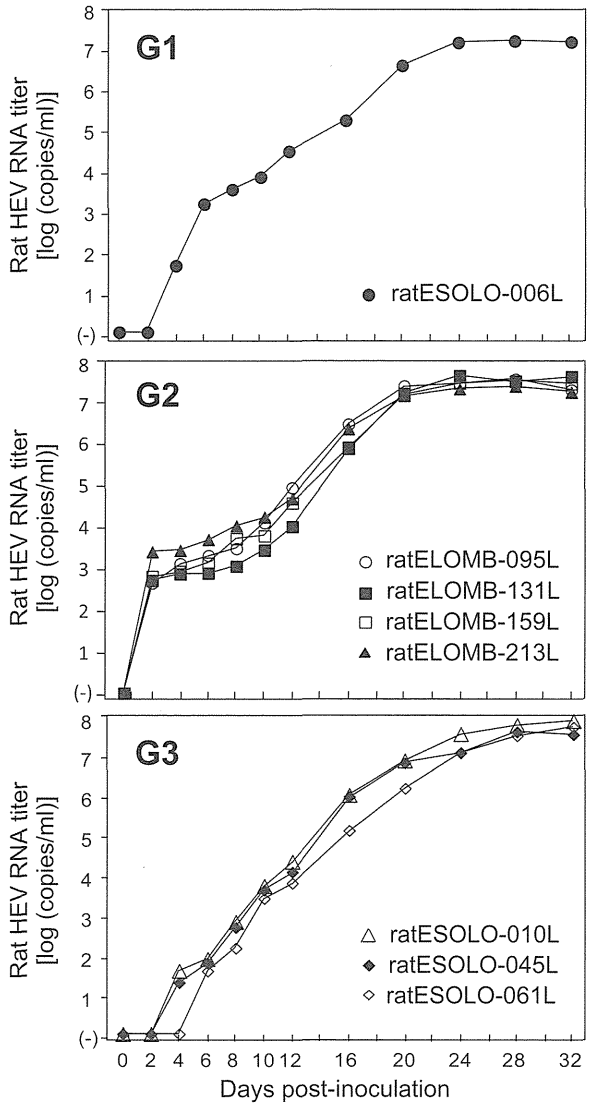

Fig. 1. Quantitation of rat HEV RNA in the culture supernatant of PLC/PRF/5 cells inoculated with liver homogenates of the indicated rat HEV strains belonging to the various genetic groups (G1–G3).

incorporated into the reverse primer. The amplified PCR product was gel purified using the FastGene gel extraction kit (Nippon Genetics Co., Ltd., Tokyo, Japan) and cloned into the *Sal*I- and *Bam*HI-linearized vector pUC19 (TaKaRa Bio, Shiga, Japan). Transcription and labeling of the negative-strand rat HEV RNA *in vitro* were performed using the AmpliScribe™ *T7-Flash*™ Transcription Kit (Epicentre, Madison, WI) according to the manufacturer's instructions.

2.8. Northern blotting analysis

The Northern blotting analysis was performed according to the previously described method (Kobayashi et al., 2011; Mikami et al., 2008) with the following modifications. Total RNA extracted from the lysates of cells inoculated with rat HEV of G2 (the ratELOMB-131L or ratELOMB-213L strain) at 32 days post-inoculation (see Fig. 1) was subjected to depletion of rRNA using the RiboMinus Transcriptome Isolation Kit (Life Technologies) in accordance with the manufacturer's instructions. The resulting RNA sample (1 μg) was separated on a 1% agarose gel, and the RNA in the gel was transferred onto a Hybond-N nylon membrane (GE Healthcare, Turnpike Fairfield, CT). After being treated in a pre-hybridization buffer, the membrane was then hybridized under high stringency conditions at 68 °C overnight in a solution containing a DIG-UTP-labeled RNA probe (50 ng/ml) that was complementary to the genomic RNA of rat HEV. After hybridization, the membrane was washed in a step-wise manner with buffers containing 0.1–2× SSPE containing 3 M sodium chloride, 200 mM sodium phosphate and 20 mM EDTA and/or 0.1% sodium dodecyl sulfate. The targeted RNA was visualized using the ImageQuant LAS500 (GE Healthcare Japan, Tokyo, Japan) with a DIG Luminescent Detection Kit (Roche Applied Science, Penzberg, Germany). The DynaMarker™ Prestain Marker for RNA High (BioDynamics Laboratory Inc., Tokyo, Japan) was used as a molecular marker.

2.9. Determination of the 5′-terminal sequence of subgenomic RNA of rat HEV in the cultured cells

In order to examine whether rat HEV possesses a subgenomic RNA encoding ORF2 and ORF3 proteins that is co-terminal with the 3′ end of the viral genomic RNA, similar to that observed in human HEV strains of genotypes 1–4, lysates of PLC/PRF/5 cells infected with rat HEV of G1, G2 or G3 were subjected to RNA extraction with TRIzol Reagent (Life Technologies) and the 5′-terminal sequence of the subgenomic RNA of rat HEV was determined using the First Choice RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion. Austin, TX), utilizing a procedure specific for capped RNA in accordance with the previously described method (Ichiyama et al., 2009). Briefly, the extracted RNA was treated with calf intestine alkaline phosphatase (CIP) followed by tobacco acid pyrophosphatase (TAP). Next, the CIP/TAP-treated RNA was ligated to a 45-nt-long RNA adapter oligonucleotide supplied in the kit by T4 RNA ligase, which was used as a template to synthesize cDNA with a rat HEV-specific antisense primer HE604 (5′-CAG CAG CGG CAC GAA CAG CA-3′; nt 4977–4996) and SuperScript II Reverse Transcriptase (Life Technologies). The cDNA was then amplified *via* nested PCR with *TaKaRa Ex Taq* (TaKaRa Bio) and the following primers: the RNA adapter outer primer supplied in the kit and RACE2 [5′-ATG AAT GAA CAC TGC GTT TGC TGG-3′] were used as first and second forward primers, respectively, while HE824 [5′-RCA CGA ACA GCA AAA GCA CG-3′; nt 4969–4988 (R = A or G)] and HE606 (5′-ACA GCA AAA GCA CGA GCA CG-3′; nt 4963–4982) were used as reverse primers for the first and second rounds, respectively. The PCR product was gel purified as described above and cloned into the pT7 Blue T-Vector (Merck Millipore). Eight independent clones were sequenced on both strands, as described above.

3. Results

3.1. Qualitative and quantitative detection of rat HEV RNA in the liver homogenate of the HEV-viremic wild rats

As described previously (Mulyanto et al., 2014), 97 (26.3%) of 369 wild rats studied had rat HEV in their sera detectable by nested RT-PCR (ORF1-PCR). When the paired liver specimens of the 97 viremic rats were tested for the presence of rat HEV RNA using ORF1-PCR, all 97 rats had detectable levels of rat HEV RNA in 10% (w/v) liver homogenate. Among the 97 infected rats, one had rat HEV of genetic group 1 (G1), while 55 had rat HEV/G2 and 41 had rat HEV/G3. Upon quantitation of the rat HEV RNA in the liver homogenates, the titer of rat HEV was 2.5×10^6 copies/ml in one rat (No. SL006) with rat HEV/G1, ranging from 8.3×10^7 to 4.8×10^8 copies/ml in the four other rats with a high titer of rat HEV/G2 and from 1.2×10^6 to 1.1×10^8 copies/ml in the three other rats with a high load of rat HEV/G3 (Table 1). These eight liver homogenate samples with

high-titer rat HEV RNA were used as inocula for cultivation of rat HEV in the four human cell lines, as described below.

3.2. Inoculation and passages of rat HEV in the human cell lines

Eight liver homogenates containing rat HEV of G1–G3 (Table 1) were inoculated onto PLC/PRF/5 cells and A549 cells in a six-well microplate at a rat HEV RNA titer of 2.4×10^5–3.2×10^7 copies/well with or without dilution at 1:3 or 1:10. Upon inoculation onto the PLC/PRF/5 cells, HEV RNA was first detected in the culture medium on the second, fourth or sixth day post-inoculation (dpi), according to the strain inoculated, then continued to increase thereafter, reaching a HEV load of $>10^7$ copies/ml at 20–24 dpi. The HEV RNA titer in the culture medium continued to be high until the observation period of 32 dpi, irrespective of the genetic group (G1–G3) of rat HEV inoculated (Fig. 1). Contrary to our expectations, the rat HEV did not produce progeny viruses in the A549 cells upon the inoculation of liver homogenate samples of five representative rat HEV strains of genetic groups G1–G3 (data not shown).

Three representative rat HEV progeny viruses of G1–G3 (ratESOLO-006L_p0, ratELOMB-131L_p0 and ratESOLO-010L_p0, respectively) released in the culture medium of PLC/PRF/5 cells, were successfully passaged in PLC/PRF/5 cells, HuH-7 cells and HepG2 cells upon inoculation at an identical HEV load of 1.0×10^5 copies/well (Fig. 2). As shown in Fig. 2, the passaged progeny viruses displayed a nearly identical replication pattern and reached a similar virus level of 7.5×10^6–1.2×10^7 copies/ml in the supernatant of the PLC/PRF/5 cells and 7.0×10^6–1.1×10^7 in the supernatant of the HuH-7 cells on 32 dpi, although the initial days of detection differed according to the rat HEV strain in the PLC/PRF/5 and HuH-7 cells. In contrast, in the HepG2 cells, the passaged progeny viruses of three representative rat HEVs (G1–G3) showed different replication patterns. The ratELOMB-131L_p0 (G2) strain replicated efficiently, reaching 3.5×10^6 copies/ml on 32 dpi, while the ratESOLO-006L_p0 (G1) and ratESOLO-010L_p0 (G3) strains propagated relatively gradually, with viral loads of 1.5×10^4 and 1.5×10^5 copies/ml, respectively, on 32 dpi. Different from that observed in the three hepatoma cell lines, the A549 cells did not support the replication of PLC/PRF/5-derived progeny viruses of any of the all three genetic groups. During these cultivation procedures, no CPEs were seen in the cultured cells.

3.3. Comparison of nucleotide sequences between the rat HEV strains used as inocula and their progeny viruses

A phylogenetic tree constructed based on the 840-nt ORF1–ORF2 sequence, using the ferret HEV sequence as an outgroup, showed that, with respect to each of the eight strains inoculated, the wild-type virus in the serum and liver specimens and its cell culture-generated progeny virus formed a cluster with a bootstrap value of 100% (Fig. 3A). Three isolates of each strain obtained from the serum, liver homogenate and culture supernatant shared 99.8–100% nucleotide sequence identity within the 840-nt ORF1–ORF2 sequence, thereby confirming that the various rat HEV strains of the three genetic groups G1–G3 propagated successfully in the PLC/PRF/5 cells.

Progeny viruses of rat HEV strains belonging to the genetic groups of G1, G2 or G3 released from three human hepatoma cell lines, including PLC/PRF/5, HuH-7 and HepG2 cells, segregated into a cluster with each of the inoculum viruses in the liver homogenate, with a bootstrap value of 100%, confirming that the cell culture-produced rat HEVs were passaged, irrespective of the genetic group (Fig. 3B).

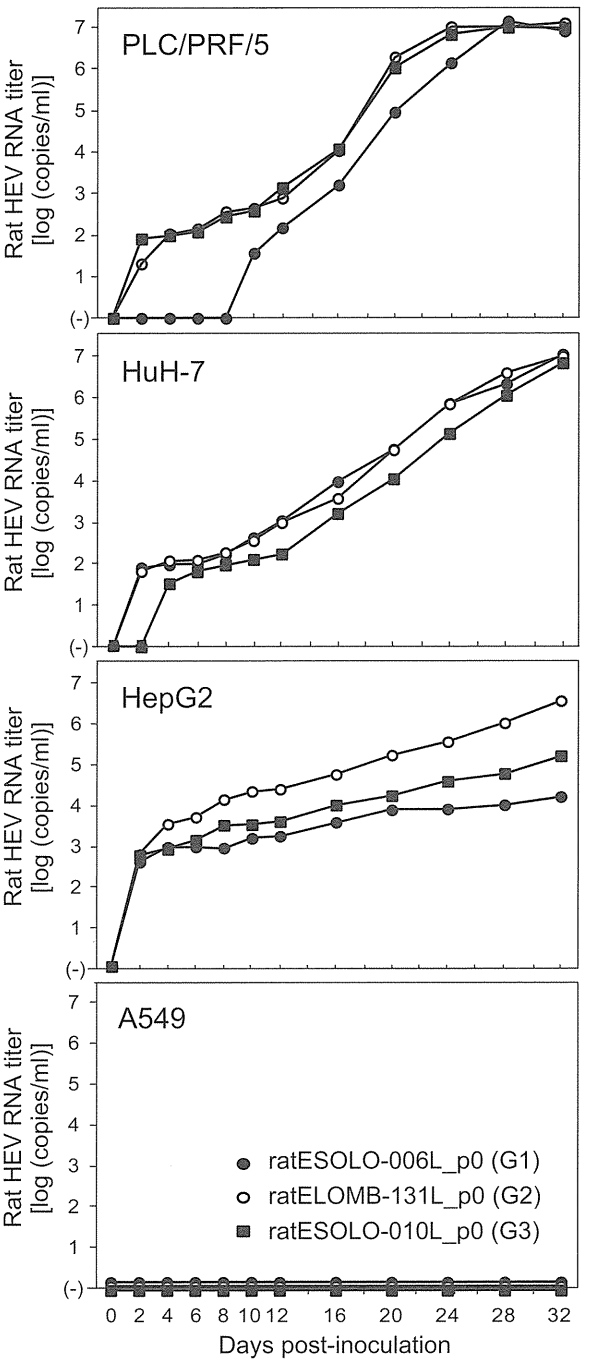

Fig. 2. Quantitation of rat HEV RNA in the culture supernatant of PLC/PRF/5, HuH-7, HepG2 and A549 cells passaged with rat HEV progeny viruses (G1–G3) originating from the culture supernatant released from PLC/PRF/5 cells.

3.4. Comparison of the buoyant density of rat HEV in the serum, liver homogenate and culture supernatant

In order to characterize the rat HEV particles, rat HEV RNA-positive serum sample (ratELOMB-131S), liver homogenates containing rat HEV (ratELOMB-131L), culture supernatant containing rat HEV progenies (ratELOMB-131L_p0) and the culture supernatant and liver homogenate samples treated with 0.5% sodium deoxycholate and 0.5% trypsin were subjected to ultracentrifugation in a sucrose density gradient. The rat HEV particles in the serum and culture supernatant banded at a density of 1.15–1.17 g/ml (Fig. 4), which was markedly lower than that of

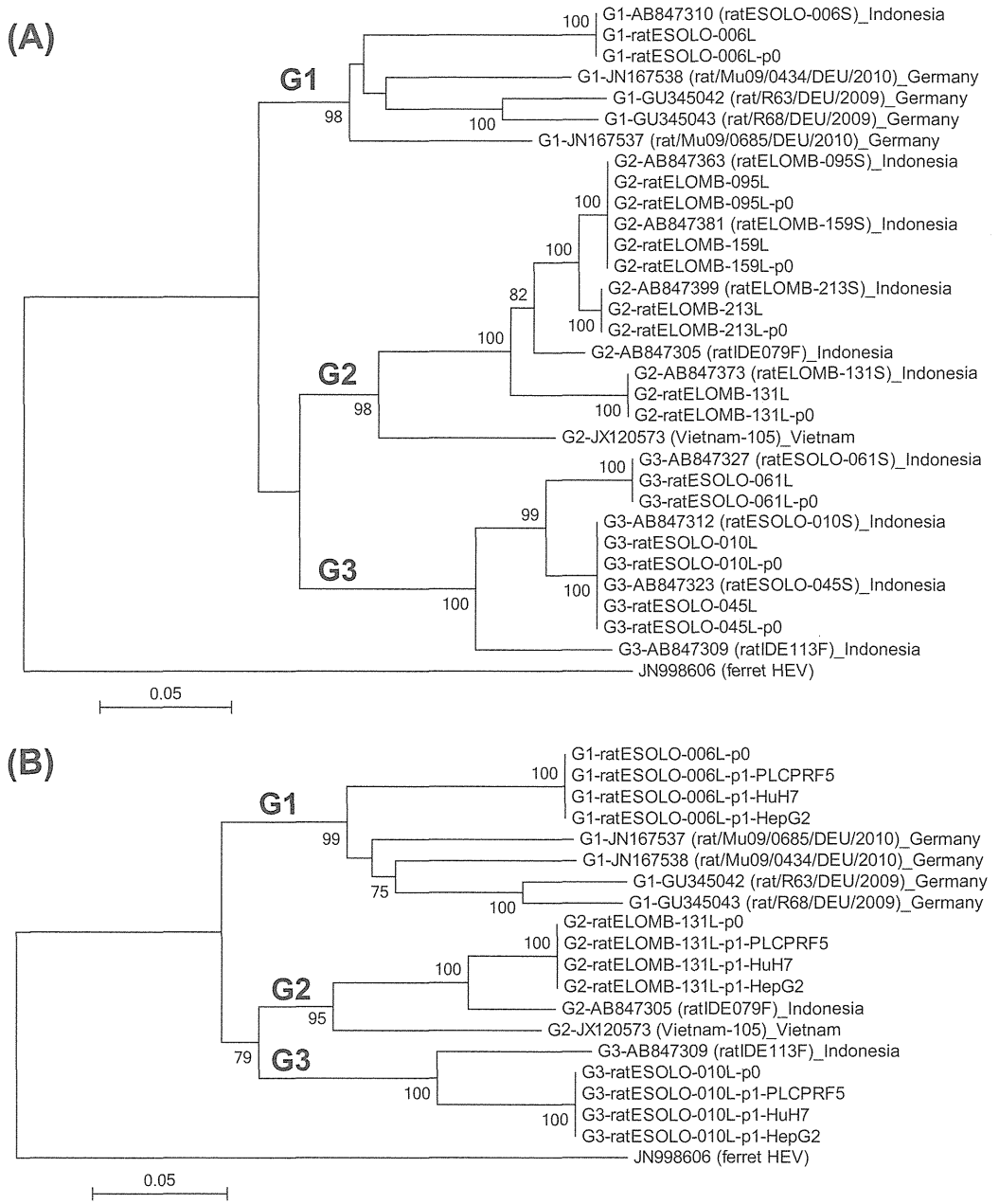

Fig. 3. (A) Phylogenetic tree constructed according to the neighbor-joining method based on the 840-nt ORF1–ORF2 sequences of 31 rat HEV isolates of genetic groups G1–G3, using a ferret HEV isolate (accession no. JN998606) as an outgroup. Seven representative rat HEV isolates whose entire genomic sequence is known are included for comparison, with the DDBJ/EMBL/GenBank accession number and isolate name given in parentheses, followed by the name of the country in which the isolate was obtained. The rat HEV isolates obtained from liver homogenate and used as the inoculum are indicated with "L", while those obtained from the culture supernatant of PLC/PRF/5 cells are indicated with "p0". (B) Phylogenetic tree constructed according to the neighbor-joining method based on the 840-nt ORF1–ORF2 sequences of 19 rat HEV isolates of genetic groups G1–G3, using a ferret HEV isolate as an outgroup. Seven representative rat HEV isolates whose entire genomic sequence is known are included for comparison. "PLCPRF5", "HuH7" and "HepG2" indicate the rat HEV isolates obtained from the culture supernatant of PLC/PRF/5, HuH-7 and HepG2 cells, respectively, which were passaged with the rat HEV progeny viruses in the culture supernatant of PLC/PRF/5 cells infected with the indicated rat HEV strains. Bootstrap values (≥70%) are indicated as the percentage of data obtained from 1000 resampling trials. Bar, 0.05 substitutions per site.

the rat HEV particles in the culture supernatant treated with sodium deoxycholate and trypsin, which peaked at 1.26–1.27 g/ml, suggesting that the rat HEV particles in the serum and culture supernatant were associated with a very-low-density material, possibly lipids. However, the rat HEV particles in the liver homogenates banded at a wide range of densities, peaking at a density of 1.13–1.15 g/ml and 1.25–1.27 g/ml, respectively. Of note, following treatment with sodium deoxycholate and trypsin, the rat HEV particles shifted to a peak density of 1.26–1.27 g/ml, suggesting the presence of viral particles with or without lipid membrane on the surface in the cell lysate, likely dependent on the stage of virion formation.

3.5. Detection of rat HEV RNA in the cell lysate using a Northern blotting analysis

To further demonstrate the successful propagation of rat HEV in human hepatoma cells and the presence of rat HEV-specific genomic and subgenomic RNA in the cultured cells, a Northern blotting analysis was performed using the DIG-UTP-labeled negative-strand rat HEV RNA probe targeting the ORF2 region (607 bp, nt 5532–6138), derived from the ratELOMB-131L strain of rat HEV/G2. As depicted in Fig. 5, two specific bands with lengths of approximately 7.0 kb and 2.0 kb, most likely corresponding to 7.2-kb genomic and 2.2 kb-subgenomic RNA of human HEV (Graff

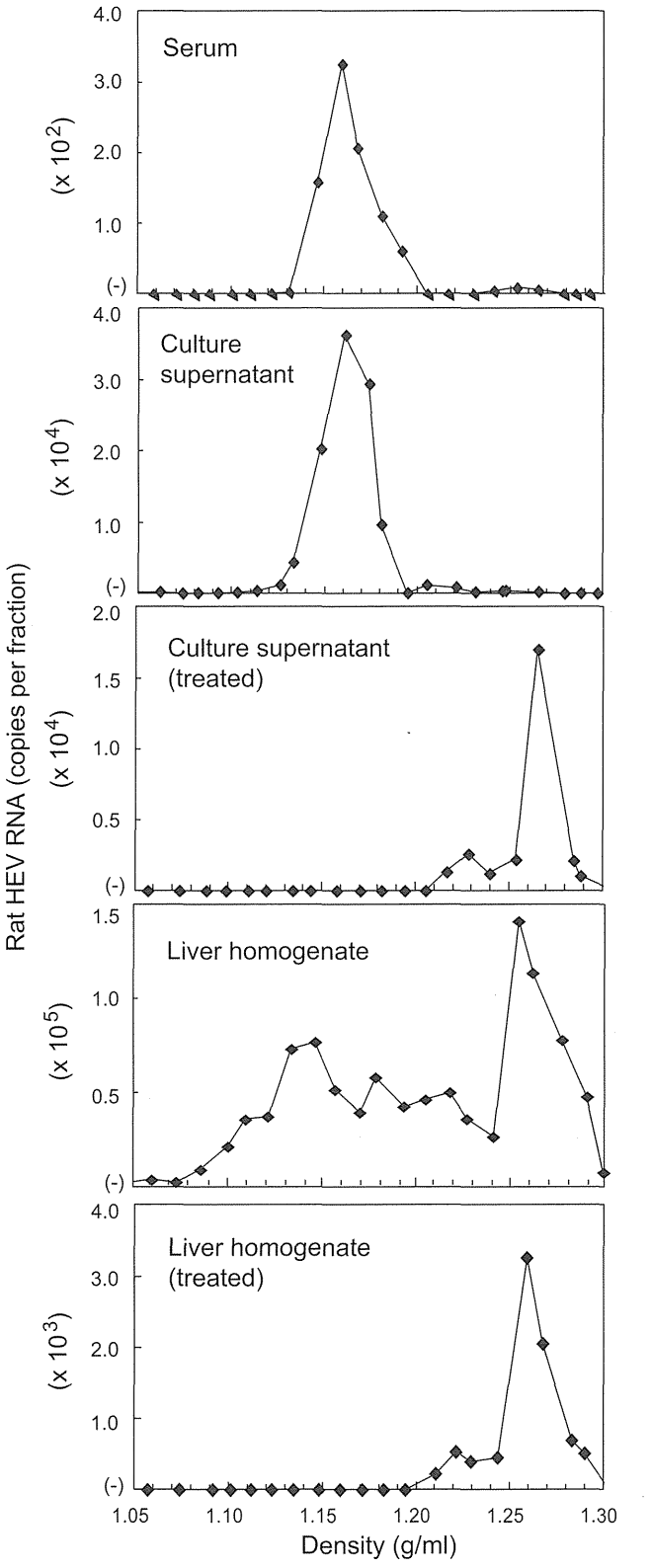

Fig. 4. Sucrose density gradient fractionation of rat HEV in the serum, culture supernatant containing cell culture-produced rat HEV and liver homogenate, as well as that treated with deoxycholate and trypsin (see Section 2.7).

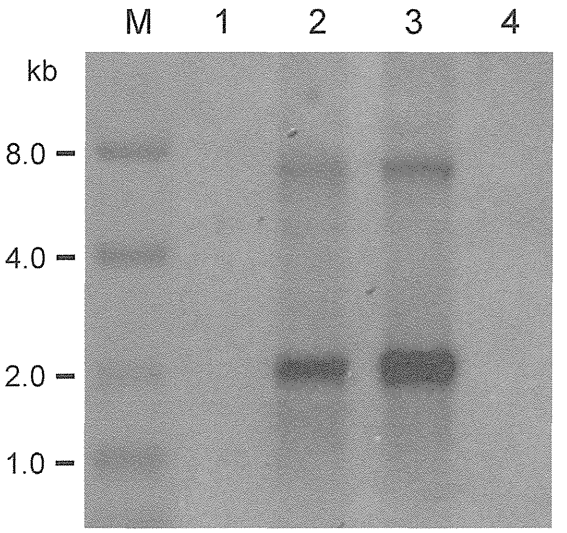

Fig. 5. Northern blotting analysis of rat HEV RNA in the cell lysates. Two rat HEV transcripts were detected using the 0.6-kb negative-strand ORF2 RNA (rat HEV/G2) as a probe. M represents RNA size markers, lanes 1 and 4 denote "mock" RNA obtained from the lysates of uninfected cells and RNA preparations obtained from the lysates of cells inoculated with cell-culture adapted human HEV (the JE03-1760F strain), as controls. Lanes 2 and 3 indicate RNA preparations obtained from the lysates of cells inoculated with two distinct rat HEV strains (ratELOMB-131L and ratELOMB-213L, respectively) of the same genetic group G2 as the RNA probe.

et al., 2006; Ichiyama et al., 2009; Tam et al., 1991), respectively, were visualized in lanes 2 and 3 [cell lysates of PLC/PRF/5 cells infected with rat HEV/G2 (ratELOMB-131L and ratELOMB-213L, respectively)]. To ensure the specificity of the analysis, mock RNA (the cell lysate of uninfected PLC/PRF/5 cells) (lane 1) and the cell lysate of PLC/PRF/5 cells infected with genotype 3 HEV (JE03-1760F) (Tanaka et al., 2007) (lane 4) were also analyzed. No specific signals were observed in lanes 1 or 4. The rat HEV RNA probe also hybridized to the genomic RNA of 7.0 kb and subgenomic RNA of 2.0 kb present in the lysates of the cells inoculated with rat HEV/G1 (ratESOLO-006L) or rat HEV/G3 (ratESOLO-010L). However, the two bands were faint, likely due to the high nucleotide sequence difference between the probe and the two rat HEV strains to be detected (19.7% or 22.7%) (data not shown).

3.6. Determination of the 5′-terminal sequence of subgenomic RNA of rat HEV in the cultured cells

In order to determine the 5′-terminal sequence of the subgenomic RNA of the rat HEV, 5′ RLM-RACE was performed, utilizing a procedure specific for capped RNA. Using nested RT-PCR with forward primers specific for the adapter and rat HEV-specific reverse primers, a single product of the expected size (approximately 80 bp) corresponding to the 5′-terminal sequence of the 2.0-kb subgenomic RNA was detected for each of the ratESOLO-006L(G1)-, ratELOMB-131L(G2)- and ratESOLO-010L(G3)-infected cell lysates. Eight clones each were obtained from the three amplification products and sequenced. A total of eight clones each from the ratESOLO-006L (G1)-, ratELOMB-131L (G2)- and ratESOLO-010L-infected cells were identical to each other within the sequence of nt 4933–4962 (the primer sequences at both ends were excluded). In addition, all 24 clones harbored the 5′-terminal sequence starting at nt 4933, and their sequences were identical to the corresponding sequence of the rat HEV obtained from the liver homogenate used as an inoculum. Of note, all 24 clones shared the extreme 5′-end sequence of GUAGC (nt 4933–4937) as well as the putative initiation codons (AUG) of ORF2 and ORF3 of the rat HEV genome located 16 nt and 5 nt downstream from the 5′ end, respectively (Fig. 6).

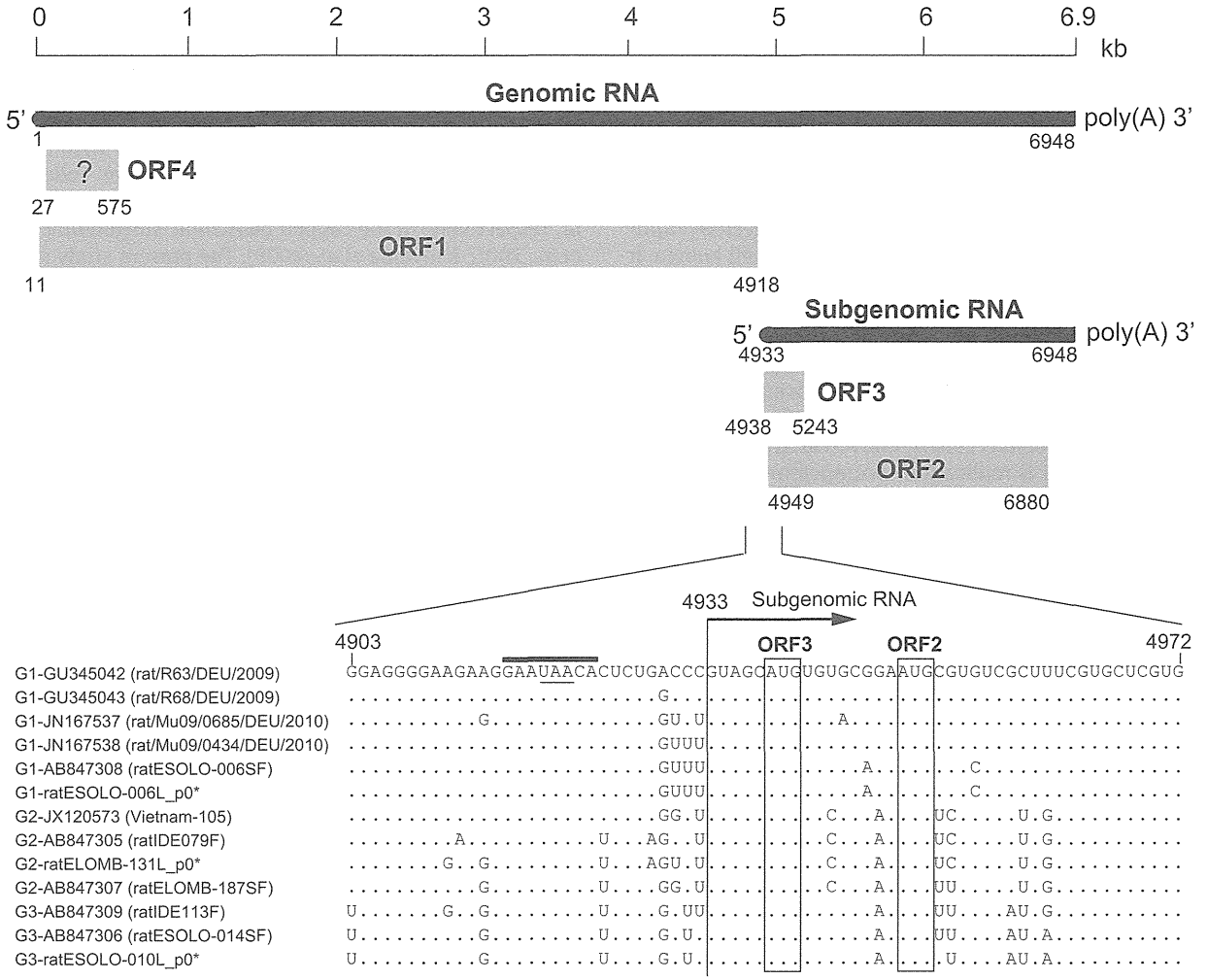

Fig. 6. Organization of the genomic and subgenomic RNA of rat HEV. The putative organization of the rat HEV genome with the locations of genomic and subgenomic RNA (closed bars) and four ORFs (shaded bars), including ORF4, whose function is unknown at present, is indicated at the top. The HEV sequences containing putative initiation codons for ORF2 and ORF3 and the initiation site of the subgenomic RNA determined in the present study are shown at the bottom. The nucleotide sequences ranging from nt 4903 to nt 4949 of representative rat HEV strains whose entire genomic sequence is known and three rat HEV strains of G1–G3 (highlighted with asterisks) for which the initiation site of the subgenomic RNA was determined in the present study were compared. The initiation site of the subgenomic RNA is indicated by a vertical bar with an arrow. Putative initiation codons of ORF3 and ORF2 are indicated by open boxes. The termination codon (UAA) of ORF1 is underlined, and a well-conserved sequence of GAAUAACA corresponding to a cis-reactive element of 12 nt (Graff et al., 2005, 2006) has a thick line drawn above it. The dots indicate nucleotides that are identical to the top sequence, and dashes denote a deletion of nucleotides. The nucleotide positions are in accordance with those of the rat/R63/DEU/2009 genome (GU345042).

4. Discussion

The present study demonstrated that rat HEV is able to replicate successfully in three human hepatoma cell lines including PLC/PRF/5, HuH-7 and HepG2 cells, regardless of the genetic group of rat HEV (G1–G3), which has been provisionally classified based on a nucleotide sequence difference of 19.5–23.5 (22.0 ± 1.7)% over the entire genome (Mulyanto et al., 2014). The rat HEV particles in the serum and culture supernatant were found to be associated with lipids, similar to human HEV particles in circulating blood and culture supernatant, which have been demonstrated to be non-neutralizable (Emerson et al., 2010; Okamoto, 2013; Takahashi et al., 2010b; Yamada et al., 2009a). Furthermore, the Northern blotting analysis indicated the presence of 7.0-kb genomic and 2.0-kb subgenomic RNA of rat HEV in the cultured cells, corresponding to two major RNA groups of human HEV, including 7.2-kb genomic and 2.2-kb subgenomic RNA (Graff et al., 2006; Tam et al., 1991), respectively.

To date, efficient cell culture systems for growing HEV in PLC/PRF/5 cells originating from human hepatocellular carcinoma and A549 cells derived from human alveolar adenocarcinoma have been developed (Okamoto, 2011a; Tanaka et al., 2007, 2009). Using these two cell lines, various HEV strains of genotypes 1, 3 and 4 obtained from clinical specimens, such as feces and circulating blood in hepatitis E patients, have been successfully propagated (Okamoto, 2011b; Takahashi et al., 2010b). Moreover, zoonotic HEV strains of genotypes 3 and 4 obtained from the serum and fecal specimens and liver homogenates of domestic pigs, wild boars and rabbits have been shown to replicate efficiently in PLC/PRF/5 and A549 cells (Jirintai et al., 2012; Okamoto, 2013; Takahashi et al., 2012). Although HepG2 and HuH-7 cells are not permissive for the wild-type genotype 3 and 4 strains of fecal origin, both of these human hepatoma cell lines are capable of supporting the successful propagation and passage of cell culture-adapted HEV strains (Okamoto, 2013). Recently, the HEV strain Kernow-C1 (genotype 3) isolated from a chronically infected patient was adapted to grow in human hepatoma cell cultures using HepG2/C3A cells (Shukla et al., 2011). The efficient multiplication of swine HEV in A549 cells has also been reported (Zhang et al., 2011). These observations indicate that various HEV strains in clinical samples obtained from hepatitis patients and from other animals, including pigs, wild boars and rabbits, can replicate efficiently in several human

hepatoma cell lines as well as A549 cells. In the present study, however, A549 cells did not support the replication of rat HEV from the liver homogenate or that of the progenies in the culture supernatant of PLC/PRF/5 cells infected with rat HEV, despite the efficient propagation of rat HEV in the three human hepatoma cell lines, including PLC/PRF/5, HuH-7 and HepG2 cells, across species barriers. The reason for the failure of rat HEV propagation in A549 cells remains unknown. Although virus entry *via* receptor-dependent clathrin-mediated endocytosis has recently been suggested (Kapur et al., 2012), the replication cycle of the HEV is not well known. It has been reported that inoculation of rat HEV into three different permanent rat liver cell lines, including N1-S1, originally isolated from a Novikoff hepatoma, the adherent cell line clone 6 from a normal liver and MH1C1 from a chemically induced hepatoma did not result in detectable virus replication, as assayed using RT-PCR with cells of the fifth passage (Johne et al., 2010). The fact that rat HEV could not be isolated in rat liver cells by one research group does not necessarily mean that it is replication-incompetent in these cells. It might be that the used specimens contained too few infectious virus particles, or the distinct procedure of infection of cell cultures was not appropriate. The analysis of the reasons for failure of rat HEV replication in A549 cells and rat liver cells deserve further analyses from various aspects in future studies in order to shed more light on its infection biology.

With regard to human HEVs, it has been demonstrated that the ORF3 protein is essential for virion egress from infected cells and is present on the surface of HEV particles (Emerson et al., 2010; Takahashi et al., 2008; Yamada et al., 2009a). The present study indicated that rat HEV particles in the serum and culture supernatant are also associated with lipids (Fig. 4). Rat HEV genomes, irrespective of the genetic group, harbor ORF3 with a coding capacity of 102 aa, similar to those (113–114 aa) of human HEV of genotypes 1–4, thus suggesting that rat HEV particles possess ORF3 proteins on their surface and that the ORF3 proteins of rat HEV also play an important role in virion release. The expression of ORF3 proteins in infected cells and the presence of these proteins on the surface of rat HEV particles in the serum and culture supernatant must be demonstrated in future studies. Recent studies have shown that an intact PSAP motif in the ORF3 protein, which is highly conserved among human HEVs of all four genotypes and even avian HEV strains, is required for the formation and release of membrane-associated HEV particles, utilizing host proteins associated with endosomal-sorting complexes required for transport (ESCRT) (Emerson et al., 2010; Nagashima et al., 2011a, 2011b; Yamada et al., 2009a). The hijacking of host-derived membranes by another enterically transmitted hepatitis virus, hepatitis A virus, with their biosynthesis being dependent on ESCRT components, has also been reported (Feng and Lemon, 2013; Ramakrishnaiah and Van Der Laan, 2013). Of interest, however, the putative ORF3 protein of rat HEV lacks the P(T/S)AP late-domain, although it possesses a proline-rich sequence of PXYPMP (aa 93–98) at its C terminus that is well conserved among all rat HEV strains thus far isolated. It deserves further analysis to clarify whether the PXYPMP sequence is involved in the formation and budding of membrane-associated rat HEV particles.

Tam et al. (1991) reported the existence of two 3′ co-terminal subgenomic RNAs of 2.2 and 3.7 kb in the liver of a macaque infected with genotype 1 human HEV. However, consistent with the finding of later studies (Graff et al., 2006; Ichiyama et al., 2009), no RNA signals corresponding to the 3.7-kb subgenomic RNA were detectable in the Northern blotting analysis of rat HEV RNA in the cultured cells in the present study, suggesting that the presence of two RNA species of genomic and subgenomic RNA is one feature common to hepeviruses. In the present study, the 5′-terminal sequence of a 2.0-kb subgenomic RNA, possessed in common by the rat HEV strains of the three genetic groups, was determined and found to start at nt 4933 with the sequence of GUAGC. It has been demonstrated that the ORF2 and ORF3 proteins of genotype 1, 3 and 4 human HEVs are encoded by a 2.2-kb subgenomic RNA (Graff et al., 2006; Ichiyama et al., 2009), and that the capped subgenomic RNA initiates exclusively at nt 5122 (Sar-55 isolate: GenBank/EMBL/DDBJ accession no. AF444003) with the common sequence of 5′-GC, while the authentic initiation codons (AUG) of ORF3 and ORF2 are located just after short UTRs of 9 nt and 20–23 nt, respectively, in the capped bicistronic subgenomic RNA (Graff et al., 2006; Ichiyama et al., 2009). The present study showed that the initiation codons of the putative ORF3 and ORF2 of rat HEV are present after even shorter UTRs of 5 nt and 16 nt, respectively, in the 2.0-kb capped subgenomic RNA. By exploring the functional consequences of unusually short 5′ UTR sequences on eukaryotic mRNA, it has been reported that, as the distance of the first AUG codon from the m7G cap decreases from 32 to 3 nt, the yield of proteins initiated from the first AUG codon progressively decreases, with a corresponding increase in initiation from the second AUG codon (Kozak, 1991). Therefore, it is very likely that, although the 5-nt UTR for ORF3 is too short, both ORF3 and ORF2 in the subgenomic RNA of rat HEV are functional and capable of encoding 102 aa and 644 aa, respectively, due to the leakiness of ribosomal scanning attributable to an extremely short leader sequence (Kozak, 1991). Infectious cDNA clones of human HEV strains, such as JE03-1760F (Yamada et al., 2009b) and Kernow passage 1 (Shukla et al., 2012), that can replicate efficiently in cultured cells have been constructed. The development of a reverse genetics system for rat HEV that is usable in the cell culture systems established in the present study will be useful for elucidating the mechanism of rat HEV replication as well as the functional roles of rat HEV proteins. Different from human HEV genomes consisting of three ORFs (ORF1–ORF3), putative ORF4 capable of encoding 183 aa is present in all known rat HEV genomes (Johne et al., 2012; Li et al., 2013a; Mulyanto et al., 2014) and is also found in ferret HEV genomes (Raj et al., 2012). The development of a reverse genetics system for rat HEV will also be useful for clarifying whether ORF4 plays an important role in viral replication and infectivity *in vitro* and *in vivo*.

A weakness of the present study is the lack of demonstration of rat HEV particles or expressed virus proteins as all analyses were exclusively based on the detection of the rat HEV genome. Therefore, the production of specific antibodies against rat HEV ORF2 and ORF3 proteins and the visualization of virus particles in the supernatants of the cell cultures by electron microscopy are needed in future studies. By electron microscopy, the presence of membrane structures on the virus particles should also be confirmed.

In conclusion, cell culture systems for rat HEV were developed in the present study. The successful propagation of rat HEV in human hepatoma cell lines observed in the current study and the presence of antibodies against rat HEV in human sera reported by Dremsek et al. (2012) raise concern regarding the potential of the zoonotic transfer of rat HEV into humans. Further investigation is needed to better understand the epidemiology, infection biology and clinical manifestations of rat HEV infection in rats and humans. The production of specific antibodies against rat HEV proteins, the clarification of the reason for the failure of rat HEV propagation in A549 cells and the development of a reverse genetics system using an infectious cDNA clone of rat HEV that can replicate efficiently in the robust cell culture systems would facilitate further research regarding the mechanisms underlying the viral replication and functional roles of rat HEV proteins.

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Case Report

High genomic similarity between European type hepatitis E virus subgenotype 3e strains isolated from an acute hepatitis patient and a wild boar in Mie, Japan

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A 67-year-old male living in Tsu city, Mie prefecture, Japan was referred to our hospital for further examination of acute liver injury and was diagnosed as having clinical hepatitis E virus (HEV) infection in January 2010. The HEV strain (HE-JA11-1701) isolated from the patient belonged to genotype 3 and European-type subgenotype 3e. It was presumed that the patient had been infected from a wild boar (*Sus scrofa leucomystax*) because he consumed meat/viscera from a wild boar that he had captured himself as a hunter approximately 2 months before disease onset. A specimen of the boar meat/viscera that the patient had ingested was not available. However, the HE-JA11-1701 strain was 99.8% identical within the 412-nucleotide sequence of the open reading frame 2 region to a HEV strain (JBOAR012-Mie08) that had been recovered from a wild boar captured near the patient's hunting area in 2008. A phylogenetic analysis confirmed that the two HEV strains had a close genetic relationship and were segregated into subgenotype 3e, supported by a high bootstrap value of 99%. Of note, the HE-JA11-1701 and JBOAR012-Mie08 strains were remotely related to the 3e strains reported in Japan and European countries, with a nucleotide difference of 7.9–13.9%, reinforcing the uniqueness of the 3e strains obtained in the present study. These results strongly support our speculation that the patient developed acute hepatitis E via consumption of HEV-infected boar meat/viscera. Genetic analyses of HEV strains are useful for tracing infectious sources in sporadic cases of acute hepatitis E.

Key words: hepatitis E virus, Japan, nucleotide sequence, subgenotype 3e, wild boar

INTRODUCTION

HEPATITIS E VIRUS (HEV) is transmitted via the fecal–oral route through the consumption of contaminated water or food. HEV infection had long been considered to be restricted to developing countries where sanitation conditions are suboptimal. However, HEV is now recognized to be an important pathogen of acute hepatitis in industrialized countries. Both HEV and hepatitis caused by HEV appear to exist virtually everywhere worldwide.[1] In Japan, autochthonous HEV strains were first recovered in 2001 from a Japanese patient with sporadic acute hepatitis E who had no history of traveling abroad and from domesticated pigs independently.[2,3] Zoonotic food-borne transmission of HEV via the ingestion of meat or viscera of infected animals including pigs, wild boar and deer is the main route of HEV transmission in Japan,[4–6] where transfusion-transmitted HEV infection is reported to rarely occur.[7,8] However, infectious source(s)/route(s) remain unknown in nearly half of hepatitis E cases in Japan.[4]

Four major genotypes of HEV that infect humans have been identified thus far.[9] Unlike genotypes 1 and 2 that are responsible for the majority of HEV infections in

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developing countries, both genotypes 3 and 4 cause acute sporadic hepatitis in industrialized countries and some developing countries and exhibit the characteristics of zoonosis. Pigs are the most frequent HEV reservoir among animals in Japan.[10,11] HEV has been isolated from the serum and liver specimens of wild boars,[12,13] and many cases of hepatitis E that developed after the patients ate wild boar meat have been reported,[14–21] with direct evidence of HEV infection occurring via the consumption of boar meat.[14] Hence, it is beyond doubt that wild boars serve as another important reservoir for HEV in humans.

Autochthonous HEV strains obtained from humans and animals in Japan belong to genotype 3 or 4, and Japan-indigenous genotype 3 HEV strains have been provisionally classified into three subgenotypes: 3b (3jp), 3a (3us) and 3e (3sp), where "jp" stands for Japan-type, "us" for US-type and "sp" for Spanish (European) type.[6,22,23]

Recently, we experienced a male patient from Mie prefecture, Japan, with sporadic acute hepatitis E (Fig. 1). The patient used to hunt wild boars as a hobby and would consume the meat/viscera of the captured boars. Because there were no leftovers of the boar meat and viscera that the patient had ingested before the onset of disease, the nucleotide (nt) sequence of the HEV strain recovered from the patient was compared with that of a HEV strain obtained from an infected wild boar that had been captured near the patient's hunting area in Mie prefecture. Based on a high genomic similarity between the HEV strains of the rare subgenotype 3e obtained from the patient and the wild boar, we herein report the case of a hunter who developed sporadic acute hepatitis E, most likely via the consumption of meat/viscera from a captured boar.

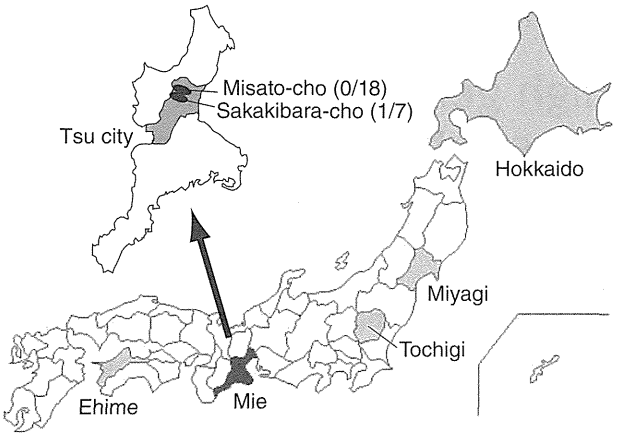

Figure 1 Map of Japan showing five prefectures where the 3e hepatitis E virus (HEV) strains have been identified and a magnified map of Mie prefecture indicating Tsu city with two hunting areas (Misato-cho and Sakakibara-cho). A total of 25 wild boars were captured during 2008–2009, and their serum samples were subjected to HEV RNA detection. Misato-cho was the patient's hunting area and Sakakibara-cho was the area where the wild boar (JBOAR012-Mie08) was captured. Between these two neighboring regions, there are no obstacles, such as large rivers, mountains or roads, that would prevent wild boars from movement. The numbers before and after the slashes in parenthesis indicate the numbers of samples positive for HEV RNA and those that were tested for the presence of HEV RNA in the present study, respectively.

CASE REPORT

IN JANUARY 2010, a 67-year-old man was referred to our hospital with a clinical diagnosis of acute liver injury. He had no symptoms at the referral visit; however, his laboratory data revealed an elevation of the serum liver enzyme levels (aspartate aminotransferase, 616 IU/L; alanine aminotransferase, 1218 IU/L; lactate dehydrogenase, 569 IU/L; alkaline phosphatase, 354 IU/L; and γ-glutamyltransferase, 94 IU/L). The prothrombin time was within the normal range (100%) and the total bilirubin level was normal (0.6 mg/dL) (Table 1). The patient had no history of traveling abroad, receiving blood or blood-related products, or injection drug use. Of note, he used to hunt wild boars as a hobby and would slaughter the boars himself. He had ingested the meat/viscera of a wild boar captured at the Misato-cho area in Mie prefecture approximately 2 months before the onset of acute hepatitis (Fig. 1). Although the viral markers of hepatitis A, B and C were negative in the serum, the patient had the immunoglobulin (Ig)M, IgA and IgG classes of anti-HEV antibodies detectable by an in-house enzyme-linked immunoassay with recombinant open reading frame 2 (ORF2) protein,[24] as well as HEV RNA detectable by nested reverse transcription polymerase chain reaction with primers targeting the ORF2 region,[5] which led to the diagnosis of acute hepatitis E. The patient recovered within 1 month after disease onset without any sequelae.

The 412-nucleotide (nt) sequence within the ORF2 region of the HEV strain (HE-JA11-1701) recovered from the patient was identified using a previously described method,[5] and deposited to the GenBank/EMBL/DDBJ databases under accession no. AB795981. The HE-JA11-1701 strain belonged to genotype 3 and was most similar to European type subgenotype 3e HEV strains, with an identity of 86.4%, 91.0% and

Table 1 Laboratory data at presentation to our hospital

Hematology		Electrolytes and renal function	
WBC	$3960 \times 10^3/\mu L$	Na	140 mEq/L
RBC	$485 \times 10^6/\mu L$	K	4.4 mEq/L
Hemoglobin	14.7 g/dL	Cl	100 mEq/L
Hematocrit	45.5%	BUN	13 mg/dL
Platelet	$220 \times 10^3/\mu L$	Creatinine	0.82 mg/dL
Neutrophil	63%	Uric acid	6.0 mg/dL
Lymphocyte	27%		
Monocyte	5%	Blood coagulation	
Eosinophil	4%	Prothrombin time	100%
Basophil	1%		
Blood chemistry		Virus markers	
T-Bil	0.6 mg/dL	IgM-HAV Ab	(−)
D-Bil	0.2 mg/dL	HBsAg	(−)
AST	616 IU/L	HCV Ab	(−)
ALT	1218 IU/L	IgG anti-HEV Ab	0.513 (+)
LDH	569 IU/L	IgM anti-HEV Ab	1.568 (+)
γ-GT	94 IU/L	IgA anti-HEV Ab	1.833 (+)
ALP	354 U/L	HEV RNA	(+)
LAP	91 IU/L		
Ch-E	278 IU/L	Autoantibodies	
Total protein	7.2 g/dL	ANA	(−)
Albumin	4.5 g/dL		
T-chol	196 mg/dL		
Triglyceride	50 mg/dL		
Blood sugar	137 mg/dL		

γ-GT, γ-glutamyltransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA, antinuclear antibodies; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ch-E, cholinesterase; D-Bil, direct bilirubin; HBsAg, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; IgM HAV Ab, immunoglobulin M-hepatitis A antibody; LAP, leucine aminopeptidase; LDH, lactate dehydrogenase; RBC, red blood cell count; T-Bil, total bilirubin; T-chol, total cholesterol; WBC, white blood cell count.

91.3% to the representative isolates of subgenotype 3e whose entire genomic sequences have been determined: HE-JA04-1911, swJ8-5 and swJ12-4, respectively.[25] With regard to the 26 other 3e strains reported in Japan (see Fig. 2 for the accession numbers), the HE-JA11-1701 strain shared only 86.1–91.0% identity within the overlapping 412-nt sequence. Because none of the boar meat/viscera that the patient had ingested before the onset of hepatitis remained, we attempted to detect HEV RNA in stored serum samples of 25 wild boars that had been captured around the patient's hunting area during the hunting season of autumn 2008 through spring 2009. The 25 serum samples were assessed for the presence of HEV RNA. Notably, one (JBOAR012-Mie08) of seven wild boars captured in the Sakakibara-cho area tested positive for HEV RNA, while none of the 18 wild boars captured in the Misato-cho area had detectable levels of HEV RNA (Fig. 1).

The simple homology of the 412-nt sequences between HE-JA11-1701 and JBOAR012-Mie08 (accession no. AB780455) was 99.8%, with only one nucleotide substitution between these two strains. A phylogenetic tree constructed using the neighbor-joining method[26] based on the 412-nt ORF2 sequences of the two sequences (HE-JA11-1701 and JBOAR012-Mie08) with 48 reference sequences confirmed that the HE-JA11-1701 and JBOAR012-Mie08 strains are closely related mutually and form a cluster with 34 other subgenotype 3e isolates, supported by a high bootstrap value (99%) (Fig. 2). Of note, however, the HE-JA11-1701 and JBOAR012-Mie08 strains were distantly related to reported subgenotype 3e strains recovered from six cases of sporadic acute hepatitis E in Mie prefecture.[27,28] Among these six strains, the HE-JA04-1911 strain isolated from a patient living in Tsu city shared only 86.4% identity with the HE-JA11-1701 and JBOAR012-Mie08 strains within the 412-nt ORF2 sequence.