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Development and Characterization of a Genotype 4 Hepatitis E Virus Cell Culture System Using a HE-JF5/15F Strain Recovered from a Fulminant Hepatitis Patient▽

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We developed an efficient cell culture system for genotype 4 hepatitis E virus using the HE-JF5/15F strain recovered from a fulminant hepatitis patient. The sixth-passage virus in the culture supernatant reached 1.5 × 10^8 copies/ml at 10 days postinoculation and possessed 10 nucleotide mutations with four amino acid changes.

Hepatitis E virus (HEV) is an important human pathogen responsible for acute or fulminant hepatitis E (2, 4, 25, 32). HEV is currently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (3). It is a single-stranded, positive-sense, polyadenylated RNA molecule of approximately 7.2 kb in size with short 5′ and 3′ untranslated regions (37, 41). The viral genome consists of three open reading frames (ORFs). ORF1 encodes nonstructural proteins involved in virus replication and virus protein processing. ORF2 encodes a 660-amino-acid (aa) capsid protein. ORF3 encodes a small multifunctional protein (113 or 114 aa) that is essential for viral infectivity in vivo (10, 40). Both the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA (7). Four major genotypes (genotypes 1 to 4) of HEV have been identified in mammalian species. Genotype 1 and 2 HEVs were responsible for waterborne epidemics of HEV infection in Asia, Africa, and Latin America. Genotype 3 HEV has been found worldwide, and genotype 4 was isolated in Asia (18, 26, 30). Comprehensive molecular and serological data have led to the consensus that hepatitis E is a zoonosis with a reservoir in pigs and possibly a range of other mammals (8, 19–21, 28, 31, 33, 34, 39, 43). Genotype 3 and 4 HEVs are considered to undergo zoonotic transmission (18, 26, 30).

Recently, using a fecal suspension with a high HEV load (2.0×10^7 copies/ml) as an inoculum, originally obtained from a Japanese patient who contracted a domestic infection with a genotype 3 HEV (the JE03-1760F strain), we developed an efficient cell culture system for HEV in PLC/PRF/5 and A549 cells, which yielded a maximum HEV load of 10^8 copies/ml in the culture supernatant, and successfully propagated 13 generations of serial passages of culture supernatant (17, 38).

In the present study, we developed a more efficient cell culture system for HEV in PLC/PRF/5 and A549 cells, using a fecal suspension with a high load (1.3×10^7 copies/ml) of genotype 4 HEV obtained from a Japanese patient with fulminant hepatitis E, and compared its replicative ability in cultured cells with those of the JE03-1760F strain and its cell culture-generated variants. Next, we characterized the changes that occurred in the HEV RNA sequence during consecutive passages in A549 cells.

With informed consent, fecal samples were periodically collected from a 58-year-old Japanese man who contracted a domestic infection with a genotype 4 HEV (HE-JF5 strain) and developed fulminant hepatitis E in 2002 (35, 43). A hepatocarcinoma cell line (PLC/PRF/5) or a lung cancer cell line (A549) (ATCC CRL-8024 and CCL-185, respectively) was grown in growth medium consisting of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen), 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B at 37°C in a humidified 5% CO_2 atmosphere, as described previously (38). Virus infection and serial passages were performed by the method described previously (38).

Total RNA was extracted from 100 μl of fecal supernatant which had been diluted with phosphate-buffered saline containing 0.1% bovine serum albumin, by using TRIzol-LS reagent (Invitrogen) or the High Pure viral RNA kit (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's instructions. Quantitation of HEV RNA was performed by real-time detection–reverse transcription-PCR according to the previously described method (38).

To determine the entire genomic sequence of HEV, total RNA was extracted from 200 μl of culture medium and subjected to cDNA synthesis followed by nested PCR of eight overlapping regions including the extreme 5′- and 3′-terminal regions as described previously (12, 24). The amplification product was sequenced on both strands directly or after molecular cloning, using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequence analysis was performed using Genetyx-Mac 12.2.7 (Genetyx Corp., Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) (11).

A fecal suspension containing the HE-JF5/15F strain with the highest HEV load (1.3×10^7 copies/ml), collected on the 15th day after disease onset, was inoculated on fresh monolay-

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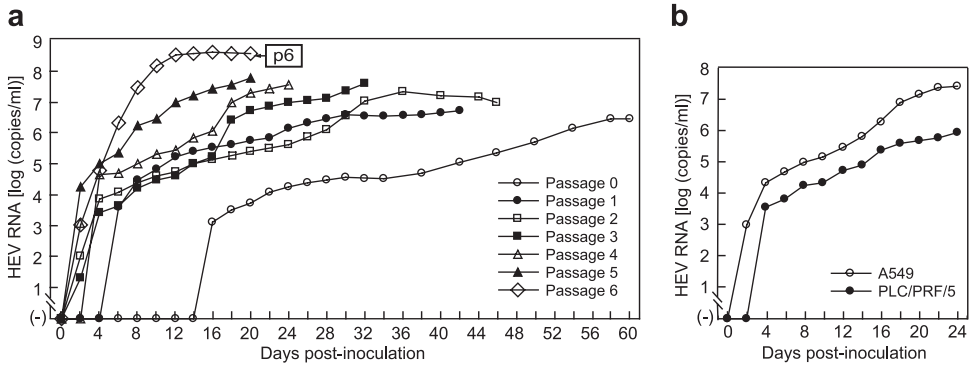

FIG. 1. Quantitation of HEV RNA in culture supernatants of PLC/PRF/5 cells inoculated with fecal supernatant (2.6×10^5 copies per well) (passage 0) or culture medium (4.3×10^4 copies per well) of passage 0 or A549 cells inoculated with culture medium (1.0×10^5 to 4.8×10^5 copies per well) of passage 1, 2, 3, 4, or 5, which was harvested on the final day of each passage (42, 46, 32, 24, or 20 dpi, respectively) (a) and PLC/PRF/5 or A549 cells that were inoculated with culture medium (5.0×10^5 copies per well) of passage 1 (b). The harvested culture supernatant of each passage was purified by passage through a microfilter with a pore size of 0.22 μm and then inoculated on fresh PLC/PRF/5 or A549 cells. The boxed p6 denotes the day after inoculation when culture supernatant used for molecular cloning of the HEV genome was harvested.

ers of PLC/PRF/5 cells at a viral load of 2.6×10^5 copies per well. In the primary propagation (passage 0), HEV RNA appeared in the culture medium of PLC/PRF/5 cells on the 16th day postinoculation (dpi), with a viral load of 1.3×10^3 copies/ml, and continued to increase thereafter, with a maximum load of 2.8×10^6 copies/ml on day 60 (Fig. 1a). HEV progenies released in the culture medium were passaged six times in PRF/PLC/5 or A549 cells. HEV RNA in the first passage (passage 1) appeared at 6 dpi and reached a maximum load of 5.0×10^6 copies/ml at 42 dpi in the culture medium of PLC/PRF/5 cells. When HEV progenies in passage 1 were inoculated on PLC/PRF/5 and A549 cells, respectively, at a load of 5.0×10^5 copies per well, A549 cells supported more efficient multiplication of HEV than did PLC/PRF/5 cells (Fig. 1b). Therefore, in subsequent passages (passages 2 to 6), A549 cells were used as host cells for virus culturing in place of PLC/PRF/5 cells. In passage 2 with inoculation of the 42-dpi culture supernatant of passage 1, HEV RNA appeared at 2 dpi, reached 1.7×10^5 copies/ml at 16 dpi, and increased to 2.2×10^7 copies/ml at 36 dpi. In passages 3 to 6, HEV RNA was first detectable in the culture medium exclusively at 2 dpi and reached a load of $>10^5$ copies/ml at 4 to 14 (mean, 8.0) dpi, contrasting with passage 0, where HEV load in the culture supernatant became $>10^5$ copies/ml at 42 dpi (Fig. 1a). In comparison with the previously established cell culture system for HEV using the JE03-1760F strain where the HEV load of the cell culture-generated JE03-1760F variants in passages 5 and 10 was less than 10^7 copies/ml at 30 dpi (17), the HEV progenies in passage 6 in the present study grew markedly more efficiently, with a high HEV load of 1.5×10^8 to 3.9×10^8 copies/ml in the harvested culture medium at 10 to 20 dpi.

For detection of ORF2 protein in the culture supernatant, Western blot analysis was performed as described previously (42), using a monoclonal antibody against HEV ORF2 (H6210) (36). A single 83-kDa band was detected in 10 μl of the culture medium of passage 6 at 10 to 20 dpi (Fig. 2).

In order to examine whether the HE-JF5/15F strain was able to adapt to growth in culture of A549 cells during successive passages, the infectivities of wild-type HE-JF5/15F and its cell culture-produced variant (HE-JF5/15F_p6, called p6 hereafter for simplicity) in the 20-dpi culture supernatant in passage 6 were compared using the same viral loads of each inoculum (Table 1). Shortening of the interval between inoculation of cultures and maximum virus yield as well as increases in the yield of virus indicates adaptation to growth in cell culture. When HEV at 1.0×10^4 copies per well was inoculated, the culture supernatants yielded were negative for HEV RNA in all six wells for wild-type virus throughout the observation period of 50 days. On the other hand, upon inoculation of wild-type HE-JF5/15F at 1.0×10^5 copies per well, all six wells (100%) showed persistent virus appearance. Efficient multiplication of HEV with a load of $>10^4$ copies/ml in the culture supernatant was observed at an average of 35.0 dpi in the wild-type virus. In contrast, when p6 HEV was inoculated at 1.0×10^5, 1.0×10^4, or 3.0×10^3 copies per well, respectively, continuous growth was observed in all six wells (100%): it was seen in two (33%) of six wells with the inoculation of HEV at 1.0×10^3 copies per well. In addition, even upon inoculation of a lower dose of 1.0×10^3 copies per well, p6 showed an earlier appearance of progenies with an HEV load of $>10^4$ copies/ml in the culture supernatant than did the wild type, which had been inoculated at 1.0×10^5 copies per well (26.0 dpi versus 35.0 dpi) (Table 1). These results indicate that the wild-type HE-JF5/15F HEV adapted to growth in cell cul-

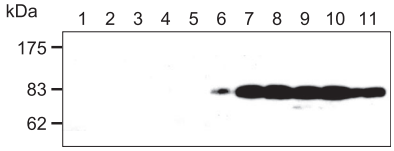

FIG. 2. Western blot analysis of ORF2 protein in the culture supernatant. At 10 to 20 dpi in passage 6, ORF2 protein in culture supernatant was detectable by Western blot analysis with an anti-ORF2 monoclonal antibody (H6210). The culture supernatant of passage 6 that was directly mixed with an equal volume of 2× sample buffer (42) was loaded at 20 μl per lane. Lane 1, 0 dpi; lane 2, 2 dpi; lane 3, 4 dpi; lane 4, 6 dpi; lane 5, 8 dpi; lane 6, 10 dpi; lane 7, 12 dpi; lane 8, 14 dpi; lane 9, 16 dpi; lane 10, 18 dpi; lane 11, 20 dpi.

TABLE 1. Comparison of infectivities of wild-type HE-JF5/15F and its cell culture-produced variant in A549 cells

Inoculum type and amt (no. of copies/well)	No. (%) of wells (n = 6) with:		Mean dpi when HEV RNA titer in culture medium reached $>10^4$ copies/ml
	Continuous HEV release	Efficient multiplication of HEV with a load of $>10^4$ copies/ml in the culture supernatant	
Fecal specimen (wild-type HE-JF5/15F)			
1.0×10^5	6 (100)	6 (100)	35.0
1.0×10^4	0	0	
3.0×10^3	0	0	
1.0×10^3	0	0	
3.0×10^2	NT[a]	NT[a]	
20-dpi culture supernatant in passage 6			
1.0×10^5	6 (100)	6 (100)	5.0
1.0×10^4	6 (100)	6 (100)	20.0
3.0×10^3	6 (100)	6 (100)	20.0
1.0×10^3	2 (33)	2 (33)	26.0
3.0×10^2	0	0	

[a] NT, not tested.

ture during six consecutive passages in our cell culture system.

Although having originated from a patient with fulminant hepatitis E, the wild-type HE-JF5/15F strain and its cell culture-generated variants did not induce a cytopathic effect in the PLC/PRF/5 or A549 cells, similar to the JE03-1760F strain (38). Persistent infection is characteristic of this virus system, like most strains of another enterically transmitted hepatitis virus, hepatitis A virus, whose cell culture system was established in 1979 (5, 9, 13, 27).

In an attempt to define the molecular mechanisms underlying the adaptation of HE-JF5/15F HEV to growth in cell culture, we determined the complete nucleotide sequence of p6 and compared the sequence of the p6 variant to the sequence of its wild-type parent, which was reported previously (12). Table 2 compares mutations over the entire genome and the predicted amino acid differences within the three ORFs between the wild-type HEV and its variant p6. The p6 isolate had the same genomic length of 7,239 nucleotides (nt) as did the wild-type HEV [the poly(A) tract at the 3′ terminus excluded]. Of note, the p6 isolate harbored 10 mutations that were restricted to ORF1 and ORF2, including a mutation at nt 6549 with the mixed nucleotide population of C as well as the U that the wild type possessed. There were five mutations at nt 1450, 2518, 4874, 6881, and 7144 in p6 that were not seen in any of the 33 reported genotype 4 HEV isolates whose entire or nearly entire sequences are available from the GenBank/EMBL/DDBJ databases as of March 2009, three of which changed amino acids (position 1617 in ORF1 and positions 565 and 653 in ORF2). The p6 HEV genome had five synonymous and two nonsynonymous substitutions in ORF1 and one synonymous and two nonsynonymous substitutions in ORF2, unaccompanied by any substitutions in the overlapping ORF3, being quite different from JE03-1760F_p13-3, which had two nonsynonymous mutations in ORF3. Of note, one of the two nonsynonymous mutations within ORF1 mapped to the RNA-dependent RNA polymerase region (aa 1617) and may be associated with heightened replication of p6 toward adaptation in culture. It has been reported that two putative stem-loop structures comprising nt 7089 to 7163 and 7173 to 7194, respectively, at the 3′ end of the HEV genome are important in concert for binding to RNA-dependent RNA polymerase (1). An A-to-G mutation at nt 7144 might be associated with heightened replicative activity. Mutations that affect the secondary structures in the 3′-terminal region can influence RNA replication (6). Therefore, synonymous mutations observed in the cell culture-generated variant in the current study may also affect viral replication and protein expression. Further studies using the reverse genetics system (42) with in vitro mutagenesis techniques are required to clarify whether the observed mutations and those that will be found in extended consecutive passages in cell culture have any drastic effects on the rate and efficiency of growth of the HEV strain HE-JF5/15F during propagation on cell cultures.

TABLE 2. Comparison of the sequences of wild-type HE-JF5 and its cell culture-produced variant (HE-JF5/15F_p6) over the entire genome

Nucleotide position	Region	Nucleotide						Amino acid	
		Wild type	p6[a]	No. of reported genotype 4 HEV strains with nucleotide[b]:				Position	Substitution
				G	A	U	C		
355	ORF1	U	C	3	9	18	3	110	
1450	ORF1	A	**U**	21	12	0	0	475	
2174	ORF1	U	C	0	1	12	20	717	Phe/Leu
2269	ORF1	A	G	10	21	1	1	748	
2518	ORF1	U	**A**	1	0	21	11	831	
3238	ORF1	A	G	2	31	0	0	1071	
4874	ORF1	G	**A**	33	0	0	0	1617	Val/Ile
6549	ORF2	U	**Y**[c]	11	0	16	6	454	
6881	ORF2	C	**U**	0	0	0	33	565	Ala/Val
7144	ORF2	A	**G**	0	33	0	0	653	Lys/Glu

[a] A progeny (HE-JF5/15F_p6) recovered from culture supernatant of passage 6. Nucleotide mutations in HE-JF5/15F_p6 that are not seen in any of the 33 reported genotype 4 HEV isolates whose entire or nearly entire sequence is known are shown in bold.

[b] A total of 33 genotype 4 HEV strains whose entire or nearly entire sequence is known have been reported, excluding the HE-JF5 isolate (AB220973), which was isolated from the serum of the present case.

[c] Y, mixture of U and C.

It is generally thought that the severity of hepatitis E depends on host factors of the infected patients such as pregnancy (14, 23) and aging (8). In addition, the presence of an underlying disease may influence the severity of hepatitis E (22). However, as with other known hepatitis viruses (15, 16, 29), recent studies suggest that HEV genotype 4 is more closely associated with the severe form of hepatitis E than is HEV genotype 3 and that a silent substitution by U at nt 3148 in genotype 4 HEV is associated with the development of fulminant hepatitis E (12, 22).

In conclusion, using a genotype 4 HEV HE-JF5/15F strain in a fecal suspension with a high HEV load of 1.3×10^7 copies/ml that was recovered from a Japanese patient with autochthonous fulminant hepatitis E, we developed an efficient cell culture system for genotype 4 HEV in A549 cells, with an HEV RNA titer of up to 3.9×10^8 copies/ml in the culture supernatant. HEV progenies released in the culture supernatant were passaged six times serially. The established HEV genotype 3 and 4 cell culture systems using the JE03-1760F and HE-JF5/15F strains, respectively, would be useful for comparative studies of the mechanism of HEV replication and functional roles of HEV proteins in relation to HEV genotype as well as the association of disease severity with HEV genotype and mutations.

Nucleotide sequence accession number. The entire nucleotide sequence of an HEV isolate (HE-JF5/15F_p6) that was determined in the present study has been deposited in the GenBank/EMBL/DDBJ databases under the accession number AB480825.

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