Expression and Self-Assembly of Empty Virus-Like Particles of Hepatitis E Virus

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Hepatitis E virus (HEV), a member of the family Caliciviridae, is one of the causative agents of viral acute hepatitis formerly known as enterically transmitted non-A, non-B hepatitis. Like hepatitis A virus, HEV is transmitted mainly by the fecal-oral route and large epidemics due to this virus are often associated with contaminated water. HEV contains a single-stranded positive-sense RNA molecule of approximately 7.5 kb that is 3′ polyadenylated and includes three open reading frames (ORFs). ORF1, located in the 5′ terminus truncated (amino acid residues 112 to 660 of ORF2), was expressed in insect Tn5 cells by a recombinant baculovirus. In addition to the primary translation product with a molecular mass of 58 kDa, a large amount of a further-processed molecule with a molecular mass of 50 kDa was generated and efficiently released into the culture medium. Electron microscopic observation of the culture medium revealed that the 50-kDa protein self-assembled to form empty virus-like particles (VLPs). The buoyant density of the VLPs in CsCl was 1.285 g/cm3 and their diameter was 23.7 nm, a little smaller than the 27 nm of native HEV particles secreted into the bile or stools of experimentally infected monkeys. The yield of the VLPs was 1 mg per 107 cells as a purified form. The particles possess antigenicity similar to that of authentic HEV particles and, consequently, they appear to be a good antigen for the sensitive detection of HEV-specific immunoglobulin G (IgG) and IgM antibodies. Furthermore, the VLP may be the most promising candidate yet for an HEV vaccine, owing to its potent immunogenicity.

In this paper, we describe the efficient expression of N-terminally truncated HEV capsid proteins derived from a Myanmar (Burmese) strain isolated in 1986 (34). The viral proteins, expressed by a recombinant baculovirus in insect Tn5 cells, were shown to be self-assembled and to form VLPs which were efficiently released into the culture medium. To our knowledge, this is the first report of high-level expression and effective formation of VLPs of HEV. According to the results of an enzyme-linked immunosorbent assay (ELISA), the particle possesses antigenicity similar to that of an authentic HEV particle and functions as an antigen to detect HEV-specific immunoglobulin G (IgG) and IgM responses. Furthermore, this molecule may be the most promising candidate yet for an HEV vaccine, owing to its potent immunogenicity.

MATERIALS AND METHODS

Construction of recombinant transfer vectors. Preparation of a pool of acute-phase stool specimens from patients with sporadic non-A, non-B hepatitis in Myanmar in 1986 and injection into rhesus monkeys (Macaca mulatta) via the intravenous route were described previously (34). Three additional passages, a total of six, were carried out, and then the bile was collected from a monkey at 7, 10, and 15 days after inoculation; the bile specimens were designated bMM41-1, bMM41-2, and bMM41-3, respectively. The total RNA was extracted with RNAzol (Biotecx Laboratories, Inc., Houston, Tex.) from bMM41-1, which contained a large number of HEV virions. The poly(A)-containing RNA was purified with oligotex-dt30(Super) (Roche Diagnostic Systems, Tokyo, Japan) according to the manufacturer’s protocols and converted into cDNA as described previously (28). Amplification of the entire ORF2 by PCR was performed with primers HEV-D2 (5′-TGGGTTCGCGAC-CAACGAGCAACC-3′) and HEV-U1 (5′-GGACACAACTGGAAGGGGGG-3′) and HEV-U2 (5′-CAACAGAAAGAAGGGGGGCA-3′) and Department of Veterinary Science, National Institute of Infectious Diseases, Japan

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2 h at 4°C, and the pellet was examined with an EM. The purified VLPs (1 Nru amplified by PCR and subcloned into plasmid pCRII to generate pHEV5134/7161. The orientation was determined by restriction enzyme digestion and sequence analyses. The 2.1-kb NruI-XhoI fragment that contains the entire ORF2, a part of the 3' noncoding region, and a sequence from the pCRII multicloning site was isolated and ligated with the baculovirus transfer vector pVL1393 to produce pVL1547/7126. The 1.6-kb DNA fragment encoding a 111-aa-truncated ORF2 (HEV nucleotide residues 5480 to 7126) was prepared by PCR with primes HEV-D13 and HEV-U14 (see Materials and Methods), which contain BamHI and XhoI sites, respectively. This was digested with the restriction enzymes and ligated into pVL1393 to generate pVL5480/7126. Symbols and abbreviations: $\triangleright$, newly introduced initiation codon; $\triangle$, termination codon; $\triangledown$, previously introduced initiation codon; B, BamHI; E, EcoRI; H, HindIII; N, NruI; X, XhoI.

**FIG. 1.** Genome organization of HEV and schematic diagram of two baculovirus transfer vectors. A DNA fragment encoding HEV nucleotides 5134 to 7161 was amplified by PCR and subcloned into plasmid pCRII to generate pHEV5134/7161. The orientation was determined by restriction enzyme digestion and sequence analyses. The 2.1-kb NruI-XhoI fragment that contains the entire ORF2, a part of the 3' noncoding region, and a sequence from the pCRII multicloning site was isolated and ligated with the baculovirus transfer vector pVL1393 to produce pVL1547/7126. The 1.6-kb DNA fragment encoding a 111-aa-truncated ORF2 (HEV nucleotide residues 5480 to 7126) was prepared by PCR with primes HEV-D13 and HEV-U14 (see Materials and Methods), which contain BamHI and XhoI sites, respectively. This was digested with the restriction enzymes and ligated into pVL1393 to generate pVL5480/7126. Symbols and abbreviations: $\triangleright$, newly introduced initiation codon; $\triangle$, termination codon; $\triangledown$, previously introduced initiation codon; B, BamHI; E, EcoRI; H, HindIII; N, NruI; X, XhoI.

CCTICTCATCCT-T-3', with the cDNA as a template. The fragments flanked by restriction enzyme sites BamHI and XhoI were ligated into the transfer vector, pVL1393, to yield plasmid pVL5480/7126. A methionine codon (underlined) for translation initiation was introduced into the forward HEV-D13 primer between the BamHI site and aa residue 112, alanine (Fig. 1).

**Generation of recombinant baculoviruses and expression of capsid proteins.** Sf9 cells (Riken Cell Bank, Tsukuba, Japan) derived from an insect, *Spodoptera frugiperda* (27), were cotransfected with linearized wild-type baculovirus pVL1393, to yield plasmid pVL5480/7126. A methionine codon (underlined) for nuclear polyhedrosis virus DNA (Pharmingen) and either *Bt*-Tn 5B1-4 (Tn5) (Invitrogen), for large-scale expression (SDS-PAGE) and analyzed by Western blot assay (17) with either serum (sMT) from a patient (patient B in reference 33) infected with hepatitis E or serum (sKFG) from a patient who developed severe icteric disease soon after entering Japan from Bangladesh in 1995. Each recombinant virus was plaque purified three times (27). Baculovirus recombinants thus obtained were designated Ac5147/7126 for the whole ORF2 and Ac5480/7126 for ORF2 truncated at the N terminus. In addition to Sf9 cells, we used an insect cell line, *Trichoplusia ni* (JRH Biosciences, Lenexa, Kan.) for 5 to 6 days at 26.5°C until an extensive cytopathic effect appeared. The multiplicity of infection (MOI) of 10 and incubated in Ex-cell 405 medium for 15 min, and further centrifuged at 10,000 $\times$ g for 30 min to remove progeny baculoviruses. The VLPs in the supernatant were concentrated by in- cubation with 8% polyethylene glycol 6000 in the presence of 0.4 M NaCl at 0°C overnight followed by centrifugation at 10,000 $\times$ g for 30 min. The VLPs were resuspended in phosphate-buffered saline (PBS) (pH 7.5), layered onto 1 ml each of 10%, 20% (wt/wt) sucrose solution, and centrifuged at 100,000 $\times$ g for 2 h at 4°C in a Beckman SW50.1 rotor. The VLPs, dispensible as a white band between the two sucrose solutions, were collected, diluted with 10 mM PBS, pH 7.2, and recovered by centrifugation at 100,000 $\times$ g for 2 h. The yield of the purified VLPs usually amounted to 1 mg per 10 7 Tn5 cells. The VLPs, discernible as a white band, were also tightly cell associated. None of these proteins self-assembled into VLPs. These findings are basically consistent with the results previously described (31, 37).

Second, in an attempt to express the capsid protein as VLPs, we prepared several mutant baculoviruses to express ORF2 proteins modified at both the N and C termini (see Discus-
One of the baculovirus recombinants, Ac5480/7126, that contains an N-terminally 111-aa-truncated ORF2 enabled us to achieve our purpose (Fig. 2, ΔORF2). Both Sf9 and Tn5 cells were infected with the virus at an MOI of 10 and incubated for 5 days. Two or three major immunoreactive proteins, including the 58- and 50-kDa proteins, were found in both cell lysates. These two proteins were detectable in the recombinant virus-infected cells, but not in mock-infected or wild-type baculovirus-infected cells, and were indistinguishable from the 58- and 50-kDa proteins found in the cells expressing the whole ORF2 sequence. The molecular mass of the 58-kDa protein was in agreement with that (59 kDa) calculated from aa residues 112 to 660 of ORF2. There was no difference between these two cells in expression, location, and tight cell association, as far as the 58-kDa protein was concerned. However, production of the 50-kDa protein differed markedly in these two cell lines. In Sf9 cells the 50-kDa protein was almost completely retained in the cells and was hard to find in the culture medium. In contrast, in Tn5 cells a large amount of the 50-kDa protein was found in the culture medium. This protein was detectable only with the serum from a hepatitis E patient (not with the normal serum).

The 50-kDa protein forms empty VLPs. The culture medium of the virus-infected Tn5 cells at 5 days p.i. was clarified by centrifugation and incubated with polyethylene glycol 6000 to see whether or not the 50-kDa protein is precipitable. After being incubated overnight on ice, the solution was centrifuged for 15 min at 10,000 × g and the supernatant and the precipitate were analyzed by SDS-PAGE. The 50-kDa proteins were recovered exclusively from the large precipitate, suggesting that the proteins were aggregated or self-assembled to form empty particles. To investigate possible particle formation by the 50-kDa molecules, the culture medium was centrifuged to remove progeny baculoviruses and directly examined with an EM (data not shown). Many round, empty, virion-like particles were observed. Most were in an aggregated form. The median size was 23.7 nm in diameter. This is a little smaller than the 27 nm of native HEV particles secreted into the bile (our observation) or the stools (2, 5, 7, 8) of experimentally infected monkeys.

Characterization of VLPs. The buoyant density of the VLPs was determined to be 1.285 g/cm³ in a CsCl gradient. Although the buoyant density of native HEV particles in the stool extract from a human volunteer was reported to be 1.35 g/cm³ and 1.39 to 1.40 g/cm³ in CsCl (5, 25), the density of the native empty virus particle is still unknown. The kinetic experiment showed that the 50-kDa protein was released into the medium at 4 days p.i. and accumulated gradually for at least another 6 days (Fig. 3). The N-terminal 15-aa sequence of the 50-kDa protein was identical to aa residues 112 to 126 of ORF2. This sequence is also the same as that of the N terminus of the primary translation product of the 58-kDa protein predicted from the plasmid construction. This indicates that the 50-kDa protein was generated from the 58-kDa protein by a posttranslational modification, probably by cleavage(s) at its C terminus. The intracellular 50-kDa protein of infected Tn5 cells also contains the same N-terminal amino acid sequence, and it was recovered as a soluble form after three cycles of freezing-thawing. This suggested that the particle formation occurred in the cells and that the physicochemical properties of the 50-kDa proteins were the same irrespective of whether they were the intracellular or extracellular form.
Antigenicity of VLPs. To determine their antigenicity, the VLPs were mixed and incubated with patient serum and the resultant antigen-antibody complexes were examined under an EM. As shown in Fig. 4C, the VLPs were coated with hepatitis E-specific antibodies, resulting in massive aggregations, whereas no aggregation was observed when the VLPs were incubated with serum from a healthy individual (Fig. 4B), indicating that the VLPs share antigenicity with authentic HEV. To further examine the antigenic specificity, the VLPs were used as the antigen to coat microplates to detect HEV-specific antibodies elicited in an experimentally infected monkey, as shown in Fig. 5A. A significant increase of both IgM and IgG antibody responses was observed during the clinical course of acute hepatitis. The IgM antibody appeared soon after the infection, reached a peak at 4 weeks, and immediately decreased. No IgM was detected after 12 weeks. The IgG response rapidly...
increased and reached a peak at 13 weeks after inoculation. The IgG titer decreased gradually, but the level, as measured by antibody ELISA, remained at an optical density of over 0.5 for at least 2 years. These results indicate that the VLPs possess at least some antigenicity similar to that of native HEV and are capable of serving as an antigen to detect HEV-specific antibodies. In order to confirm these results, the VLPs were used to detect antibodies elicited in patients with hepatitis E. Serial dilutions of sera were subjected to the antibody ELISA, and the IgG response was measured (Fig. 5B). A significant increase of reactivity was observed in the sera from patients but not in those from healthy individuals.

Immunogenicity of VLPs. Antibodies to the VLPs were prepared in rabbits and guinea pigs, as described in Materials and Methods. After injection four times, the animals had high levels of IgG antibodies and the titer reached as high as 1:10^6 as determined by the antibody ELISA. To determine the immunogenicity of the VLPs the hyperimmune serum from a rabbit was used to coat microplates and capture HEV antigens in the bile from HEV-infected monkeys or the stool specimens from patients with hepatitis E. The HEV antigen thus captured was detected by the hyperimmune serum from a guinea pig. Titers of 20 to 80 were obtained for the PCR- and EM-positive specimens but not for the negative samples. However bMM41-3, which showed the highest titer, at 320, was EM negative (Table 1). These results demonstrated that the VLPs were immunogenic and were able to elicit antibodies capable of binding to native HEV antigens.

DISCUSSION

Expression of viral structural proteins by baculovirus recombinants has long been used to generate VLPs of various DNA and RNA viruses. VLPs produced by this system usually retain the immunogenic and physicochemical properties of the native virions (9, 10, 16–18, 20, 22). As observed in this study, as well as previously (31, 37), the entire ORF2 sequence was efficiently expressed in insect cells. However, no VLPs were detected (Fig. 2). To determine the possible function of the HEV proteins encoded in ORF1 and ORF3 in self-assembly, we carried out experiments in which Ac5147/7126-infected Tn5 cells were superinfected with baculovirus recombinants that efficiently express the entire ORF1 and/or ORF3 protein. However, neither stimulation of the expression of the 72-, 58-, and 50-kDa proteins nor release of the 50-kDa protein into the medium was observed (data not shown). In contrast, the ORF2 protein truncated at its N terminus was shown to self-assemble and form the VLPs, providing direct evidence that ORF2 indeed encodes the structural protein of HEV.

Two conditions are necessary for efficient particle formation. First, the N-terminal 111 aa, including the 5\(^9\) distal leader sequence, should be deleted. Deletion of the N-terminal 15 aa was not sufficient. However, addition of approximately 30 aa derived from the pVL1393 vector to the C terminus of the truncated protein did not disrupt particle formation. In Tn5 cells, the 50-kDa protein was released into the medium while the 58-kDa protein was tightly cell associated, although these two proteins shared the same amino acid sequences at their N termini. These findings indicated that the truncated proteins

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<th>Specimen</th>
<th>Titer(^a)</th>
<th>Result of:</th>
<th>PCR(^b)</th>
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<tr>
<td>VLPs(^c)</td>
<td>1,280</td>
<td>EM</td>
<td>ND(^d)</td>
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<td>–</td>
<td>–</td>
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<td>&lt;10</td>
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<td>+</td>
<td>ND</td>
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<tr>
<td>bMM41-3(^h)</td>
<td>320</td>
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<td>ND</td>
</tr>
<tr>
<td>Stool(^i)</td>
<td>80</td>
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<tr>
<td>Stool(^j)</td>
<td>20</td>
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\(^a\) Highest dilution of the specimen.
\(^b\) Performed as described previously (2).
\(^c\) 10 ng.
\(^d\) ND, not done.
\(^e\) From a normal rhesus monkey.
\(^f\) From a healthy individual.
\(^g\) Bile from an infected monkey after passages 2 and 3, respectively.
\(^h\) From patients in China with epidemic hepatitis E (4).
should be further processed through a posttranslational C-terminal deletion mediated by host cellular protease(s) before or during self-assembly. In concert with our observation, the 55-kDa protein, a proteolytically processed ORF2 protein with its 111 aa from the N terminus of a Pakistani strain, has been shown to lack another 51 aa at its C terminus (31). Whether or not this 55-kDa and our 50-kDa protein are the same molecule is unknown.

Second, expression in Tn5, but not Sf9, cells was crucial. Sf9 cells were used exclusively for expression in the previous studies. Sf9 was indeed capable of generating both 58- and 50-kDa molecules whose N-terminal amino acid sequences are identical to that of the VLPs; however, no VLPs were formed. Although morphological changes of these two cell lines after virus infection seem to proceed similarly, as observed by optical or electron microscopy, our preliminary experiments suggested that the viability of the infected cells is an important factor for VLP formation. The biochemical basis of the prolonged viability of Tn5 cells compared with Sf9 cells and the mechanism for particle formation specific to Tn5 cells remain unknown. Release of the VLPs into the culture medium has a great advantage for obtaining a large amount of highly purified VLPs, allowing further characterization of the molecule.

Obviously the size of the VLPs (23.7 nm) is smaller than that of the authentic native HEV virion (27 nm), and the smaller HEV virions have not been observed in the bile or stools from patients with hepatitis E or from experimentally infected monkeys (25). The formation of the smaller particles described in this study is probably due to assembly of both N- and C-terminally truncated ORF2 proteins, but there is currently no collective view to explain the relationship between the processing of the capsid protein and particle formation. Recently Jameel and coworkers reported that the ORF2 protein is synthesized as an 82-kDa precursor, which is then processed into a 74-kDa mature protein through signal sequence cleavage and glycosylated into an 88-kDa protein (15). Our Western blot analysis of the bile and the stools from experimentally infected monkeys identified two major proteins with molecular masses of 50 and 58 kDa. In this context, it would be interesting to see whether or not these proteins are glycosylated. The size of our VLPs seems to be different from those found in previous studies by others, in which VLPs of 30 and 18 nm in diameter were observed by TEM using a fraction with a density of 1.30 g/ml, whereas partial purification yielded particles of approximately 20 nm (32, 37).

Nevertheless, the formation of the VLPs described here is very important for seroepidemiological studies. The VLPs appeared to retain the antigenicity of the native virion, as demonstrated by IEM with serum samples from patients with hepatitis E (Fig. 4). The antibody ELISA using the VLPs as antigen appears to be specific and sensitive enough to detect anti-HEV IgG as well as IgM in human and experimentally infected monkey sera (Fig. 5A). All these results demonstrate that VLPs are usable agents for diagnosis and may allow us to study many non-A, non-B, non-C acute hepatitis cases which have been suspected to be caused by HEV but for which obtaining proof has been hampered by the lack of reliable reagents. The virtually unlimited supply of VLPs by the baculovirus recombinant is capable of overcoming such problems. Our preliminary seroepidemiological study using VLPs indicates that the prevalence of antibody to HEV in areas in which HEV is nonepidemic is much higher than previously suspected (unpublished data). Although the neutralization test with authentic HEV particles is necessary to determine immunogenicity (23), our results indicate that the VLPs are capable of producing antibodies that specifically bind to native HEV antigen. Currently only one serotype has been found in HEV. Our data suggest that the VLPs may be the most promising molecules yet for a recombinant vaccine against HEV infection.

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