

Expression, Characterization, and Immunoreactivities of a Soluble Hepatitis E Virus Putative Capsid Protein Species Expressed in Insect Cells

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The hepatitis E virus (HEV) open reading frame-2 (ORF-2) is predicted to encode a 71-kDa putative capsid protein involved in virus particle formation. When insect *Spodoptera frugiperda* (Sf9) cells were infected with a recombinant baculovirus containing the entire ORF-2 sequence, two types of recombinant proteins were produced: an insoluble protein of 73 kDa and a soluble protein of 62 kDa. The 62-kDa species was shown to be a proteolytic cleavage product of the 73-kDa protein. N-terminal sequence analysis of the 62-kDa protein indicated that it lacked the first 111 amino acids that are present in the full-length 73-kDa protein. A soluble 62-kDa protein was produced without the proteolytic processing by inserting the coding sequence of amino acids 112 to 660 of ORF-2 in a baculovirus expression vector and using the corresponding virus to infect Sf9 cells. The two recombinant 62-kDa proteins made by different mechanisms displayed immunoreactivities very compatible to each other. The 62-kDa proteins obtained by both proteolytic processing and reengineering demonstrated much higher sensitivities in detecting anti-HEV antibodies in human sera than the antigens made from bacteria, as measured by enzyme-linked immunosorbent assay. The data suggest that the soluble 62-kDa protein made from insect cells contains additional epitopes not present in recombinant proteins made from bacteria. Therefore, the 62-kDa protein may be useful for HEV diagnostic improvement and vaccine development. The reengineered construct allows for the consistent large-scale production of the soluble 62-kDa protein without proteolytic processing.

Hepatitis E virus (HEV) is an RNA virus (26) transmitted enterically, usually through contaminated water. Numerous HEV outbreaks have occurred in developing countries in the last several decades, resulting in tens of thousands of people being infected (3, 24, 32, 43). In most cases, the clinical syndrome of infection is manifested by acute hepatitis, and the disease usually resolves without chronicity. However, the mortality from acute HEV infection ranges from 0.5% for the general population to as high as 20% for pregnant women who become infected (15, 27, 38).

Our understanding of the molecular biology of HEV replication has been hampered by the lack of a tissue culture system for virus replication. However, molecular cloning and sequence analysis of the HEV genome (2, 11, 31, 34) have revealed some possible functions of the three open reading frames (ORFs) of this virus. ORF-1 is predicted to be a polycistronic protein containing five domains, including those for methyltransferase and RNA-dependent RNA polymerase (8, 17, 27, 28). ORF-2 is presumed to encode a capsid protein with a predicted molecular size of 71 kDa (2, 11, 31, 34). The function of the small polypeptide of 123 amino acids encoded by ORF-3 is not clear, but the sequence contains an immunodominant epitope recognized by acute- and convalescent-phase sera of infected patients, suggesting a potentially important function (14, 40).

Substantial progress has been made in characterization of recombinant antigens for HEV diagnostics and vaccine development. Recombinant proteins from ORF-2 of HEV have been shown to be reactive by Western blotting (immunoblotting) and enzyme-linked immunosorbent assay (ELISA) with

human sera from well-defined cases of hepatitis E and with sera from primates experimentally infected with HEV (7, 10, 25, 37, 40). Immunodominant epitopes in ORF-2 and ORF-3 have been included in commercially available diagnostic ELISAs for HEV (5, 6, 13, 14, 40, 41). The establishment of an animal model of HEV infection, in cynomolgus macaques (4, 20, 33, 35), made the evaluation of vaccine candidates possible. In a previous study, immunization of cynomolgus monkeys with a truncated ORF-2 from HEV strain Burma expressed in *Escherichia coli*, trpE-C2, elicited a protective immune response against homologous wild-type HEV challenge (25); however, virus shedding was not prevented when vaccinated animals were challenged with a heterologous strain of HEV. In a subsequent study, a recombinant ORF-2 protein species produced in insect cells showed promising results after single and double vaccination in offering protection from HEV disease (36, 37). We have concentrated on the expression of HEV capsid antigen in eukaryotes such as in insect cells using recombinant baculovirus (21, 29) since many viral immunological epitopes such as those from the viral capsid and outer membrane are highly conformational and may require posttranslational modifications such as glycosylation (1, 12, 16, 18, 19). In this report, we describe our effort to fully characterize the recombinant HEV ORF-2 protein species produced in insect cells. Expression of the full-length ORF-2 under different conditions could produce two distinct species, an insoluble 73-kDa protein and a soluble 62-kDa protein that was dependent on a proteolytic cleavage process. The 62-kDa species demonstrated improved sensitivity in anti-HEV immunoglobulin M (IgM) detection, suggesting that it may be useful for early HEV diagnostics and vaccine development as well.

A strategy for efficiently expressing this soluble immunore-

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active protein without proteolytic processing was developed, making large-scale production and purification possible.

MATERIALS AND METHODS

Insect cells. *Spodoptera frugiperda* (Sf9) suspension cells were a generous gift from Robert Lanford of Southwest Foundation for Biomedical Research (San Antonio, Tex.). The Sf9 monolayer cell line was kindly provided by Sergei A. Tsarev of the National Institutes of Health (Bethesda, Md.). Both cell lines were maintained at 27°C in Grace's insect medium (catalog no. 11605-011; Gibco/BRL) supplemented with 5% fetal bovine serum and 50 µg of gentamicin (catalog no. 15710-015; Gibco/BRL) per ml. In addition, 0.1% Pluronic F-68 (catalog no. 24040-016; Gibco/BRL) was used in suspension cell cultures.

Recombinant baculoviruses. Recombinant baculovirus ORF-2-rAcNPV expressing the entire ORF-2 of the Burma strain of HEV was constructed as described previously (10). For the construction of the recombinant baculovirus vBBIII-62k for the recombinant 62-kDa (r62-kDa) protein, a 224-bp DNA fragment containing nucleotides 5480 to 5684 of the ORF-2 from the Burma strain of HEV, plus restriction endonuclease sites and a protein translation initiation codon, was synthesized by PCR with an HEV cDNA plasmid template, pBBIII-ORF-2 (30), and two oligonucleotide primers, GGG-GGG-GAT-CCA-TAT-G GC-GGT-CGC-TCC-GGC-CCA-TGA-CAC-CCC-G and ATT-AGA-AGC-TT C-CGT-GGC-CAT-TAT-ATG. *Bam*HI and *Hind*III restriction endonuclease sites were incorporated into the primers to facilitate subsequent plasmid construction. The PCR product was digested with *Bam*HI-*Hind*III and was ligated to a baculovirus expression vector, pBlueBacIII (Invitrogen, San Diego, Calif.), that had been digested with the same restriction endonucleases to form the plasmid pYZ1. A 1.5-kbp *Hind*III DNA fragment, corresponding to the 3' portion of HEV ORF-2, was excised from the plasmid pBBIII-ORF-2 and was inserted into the plasmid pYZ1 at the *Hind*III site to form the final baculovirus transfer vector pBBIII-62k. The vector was recombined into the genome of a wild-type baculovirus to form the recombinant baculovirus vBBIII-62k.

Transfection, plaque purification, amplification of viral stocks, and determination of virus titers were carried out by the recommended protocol described by the reagent supplier.

Rabbit anti-HEV antisera. Expression of two HEV ORF-2-derived antigens, SG3 and 3-2(B), containing the C-terminal 327 and 48 amino acids, respectively, was described previously (41). SG3 was synthesized as a glutathione S-transferase fusion protein, while the 3-2(B) sequence was fused to a bacterial β-galactosidase gene. Polyclonal rabbit antisera were then generated by using purified antigens by hyperimmunization of the animals (30).

SDS-PAGE and immunoblotting. To prepare protein samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), approximately 2×10^9 baculovirus-infected Sf9 cells were collected by centrifugation in a microcentrifuge and were resuspended in 150 µl of phosphate-buffered saline (PBS). The cells were lysed by sonication.

The cell lysate was subjected to centrifugation in a microcentrifuge at 4°C for 15 min. The supernatant and pellet fractions were separately denatured, followed by electrophoresis on SDS-polyacrylamide gels (10 or 12% polyacrylamide) (42). The protein gel was stained with Coomassie blue or was transferred to a nitrocellulose membrane for Western blot (immunoblot) analysis with rabbit anti-SG3 or anti-3-2(B) antisera.

Protein purification. For purification of the 73-kDa full-length ORF-2 protein, pellets of Sf9 suspension cultures infected with the recombinant baculovirus ORF2-rAcNPV were resuspended in PBS containing protease inhibitors. The suspension was lysed by passages through a Microfluidics microfluidizer (M-110S), followed by centrifugation. The pellet material was solubilized in 25 mM BICINE [N,N-bis(2-hydroxyethyl) glycine; pH 8.5] containing 0.5% SDS. The soluble fraction was chromatographed on a Hyper-D-S column in the presence of 8 M urea. The 73-kDa fractions eluted with a NaCl gradient were pooled and dialyzed against water. The precipitated material was collected and reextracted with the BICINE-SDS buffer. Dithiothreitol was added to the solution, which was then heated and diluted in a refolding buffer containing 50 mM glycine, 10% glycerol, 5 mM (each) reduced and oxidized glutathione, and 1 g of polyethylene glycol 3500 per liter. The solution was allowed to air oxidize overnight and was concentrated.

Purification of both the r62-kDa protein and the processed 62-kDa protein (p62-kDa protein) was described previously (22, 23).

ELISAs. The immunoreactivities of recombinant HEV proteins expressed in insect cells and in *E. coli* were evaluated by ELISA by the method described previously (41). Panels of coded sera from HEV-infected individuals were used to define the sensitivity and specificity of the recombinant antigens (200 ng or equivalent per well of a microtiter plate). These sera were collected mainly from various epidemics of enterically transmitted non-A, non-B hepatitis and were provided by the Centers for Disease Control and Prevention. All serum specimens except those in the endpoint titer study (see Table 3) were assayed at a 1/100 dilution with antibody diluent. Sera were tested for IgG and IgM antibodies to HEV antigens by using gamma chain or mu chain-specific conjugated antibody to human IgG or IgM, respectively. The ELISA endpoint was defined as the greatest serum dilution in a twofold dilution series that still yielded an optical density of at least 0.3.

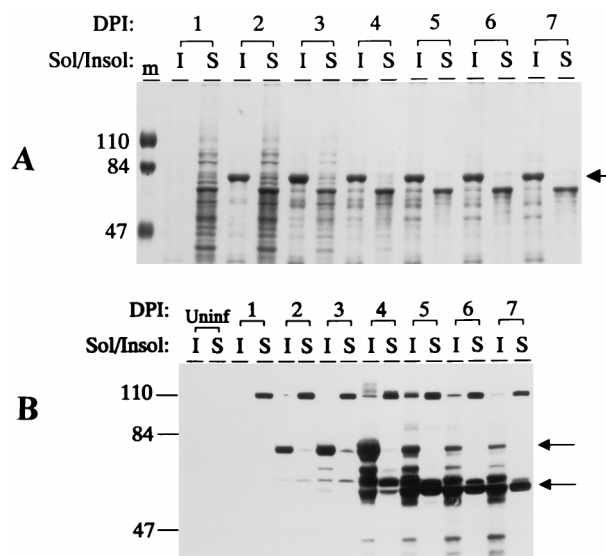


FIG. 1. Expression of HEV ORF-2 protein in insect cells using recombinant baculovirus. (A) Full-length ORF-2 protein produced in Sf9 suspension cultures. Cells infected with a recombinant baculovirus expressing the full-length ORF-2 of the HEV Burma strain were collected at various days postinfection (DPI). Cell lysates were prepared by sonication in the presence of PBS. A centrifugation process was used to separate the soluble (lanes S) from insoluble (lanes I) materials, and both were applied to SDS-polyacrylamide gels, followed by Coomassie blue staining, and both were applied to SDS-polyacrylamide gels, followed by Coomassie blue staining. An arrow indicates the full-length recombinant ORF-2 protein. Lane m, protein molecular size standard (numbers on the left are in kilodaltons). (B) Western blot analysis showing the production of a soluble p62-kDa protein in separate Sf9 monolayer cell lines. The experimental protocol was essentially the same as that described for panel A except that immunoblotting was carried out with rabbit anti-3-2(B) antiserum. Arrows indicate the 73-kDa (upper arrow) and p62-kDa (lower arrow) proteins.

RESULTS

Expression of HEV ORF-2 in insect cells. Our initial goal was to fully characterize the HEV capsid (ORF-2) protein species expressed in insect cells. Sf9 suspension cultures were infected with a previously described recombinant baculovirus expressing ORF-2 from the HEV Burma strain (10), and the cell cultures were maintained for 1 to 7 days. Infected cells were lysed by sonication in the presence of PBS, followed by a centrifugation process to separate soluble from insoluble materials, both of which were analyzed by SDS-PAGE (Fig. 1A). A prominent protein with a molecular size of approximately 73 kDa was observed by Coomassie blue staining of the insoluble fraction of the samples obtained from 2 to 7 days postinfection. This protein was later confirmed to be the full-length ORF-2 protein by tryptic peptide analysis (data not shown). The 73-kDa protein was stable over the course of infection, because no apparent degradation was observed by SDS-PAGE analysis of sequential samples (Fig. 1A).

It was previously reported, however, that several ORF-2-related smaller proteins were observed when an Sf9 monolayer cell line was infected with a recombinant baculovirus containing the full-length ORF-2 from HEV Pakistani strain SAR-55 (37). To examine the effect of different viral strains and cell lines on the formation of ORF-2 proteins, the relevant Sf9 monolayer cell line was infected with the recombinant baculovirus used in the SDS-PAGE analysis whose results are presented in Fig. 1A. A doublet of soluble proteins of approximately 62 kDa (Fig. 1B, lower arrow) was detected late in the infection by Western blot analysis with an antiserum against ORF-2. In addition, a 110-kDa β-galactosidase polypeptide

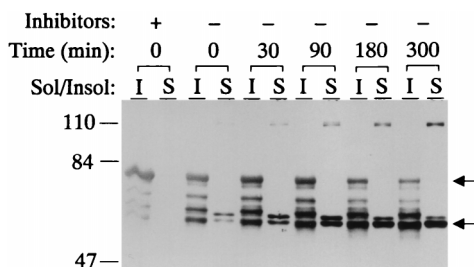


FIG. 2. Cleavage of the 73-kDa protein and formation of the p62-kDa protein in vitro. Lysates of Sf9 monolayer cells infected with recombinant baculovirus containing ORF-2 for 5 days postinfection were incubated at 22°C for various times, as indicated. PBS-soluble (lanes S) and PBS-insoluble (lanes I) materials were analyzed by SDS-PAGE, followed by immunoblotting with anti-3-2(B) antiserum. Arrows point to the 73-kDa (upper arrow) and the p62-kDa (lower arrow) proteins. The presence (+) or absence (-) of protease inhibitors in the preparations of cell lysate is indicated.

from the baculovirus vector was also detected since the antiserum was made against the HEV 3-2(B) epitope (see Materials and Methods section) fused to a β -galactosidase gene. Comparison of serial samples obtained from 2 to 7 days postinfection showed a progressive decrease in the amount of the 73-kDa protein (Fig. 1B, upper arrow), coupled with a progressive increase in the formation of the 62-kDa protein. This observation suggested that the 62-kDa protein was generated by proteolytic processing of the 73-kDa protein.

Proteolytic cleavage of the 73-kDa protein in vitro. We hypothesized that the soluble p62-kDa protein might preserve some epitopes which were otherwise destroyed by detergent solubilization of the insoluble recombinant proteins made from bacteria and insect cells as well. To evaluate the stability of the p62-kDa protein for purification, we developed an in vitro cleavage assay. Cell lysates were prepared from monolayer cultures 5 days postinfection, followed by incubation for various times at 22°C before each aliquot was analyzed for the presence of soluble p62-kDa protein (Fig. 2). Progressive disappearance of the insoluble 73-kDa protein coupled with accumulation of the typical soluble p62-kDa protein doublet was readily observed. The conversion was proportional to the time of incubation. The p62-kDa protein was fairly stable because no further degradation products were observed after 5 h of incubation. This stability was important for us to develop a protein purification protocol later (23).

Purification of the p62-kDa protein and its N-terminal sequence analysis. The soluble p62-kDa protein from monolayer cells was purified for N-terminal sequence analysis. The final purified p62-kDa protein from a Poros HQ/F column was run on a protein gel, shown in lane 2 of Fig. 3A; it ran as a single band on this low-resolution gel. The purity of the p62-kDa protein estimated by Coomassie blue staining was at least 95%.

The purified p62-kDa protein was subjected to electrophoresis, and both bands of the doublet was transferred to a polyvinylidene difluoride membrane for N-terminal sequencing by Edman degradation. The sequencing cycle versus amino acid composition of the first 12 amino acid residues is illustrated in Fig. 3B. Only one N-terminal sequence was derived; it had 11 of 12 amino acid matches (with one being unidentifiable) with the published sequence of ORF-2 (31) starting at Ala-112, indicating that the p62-kDa protein did not have the N-terminal 111 amino acids of the ORF-2. The single N terminus of the doublet indicated possible internal or C-terminal modifications of the protein. The reactivity of the p62-kDa protein with anti-3-2(B) antiserum indicated that the C-terminal cleavage, if it occurred, must be within last 48 amino acids of

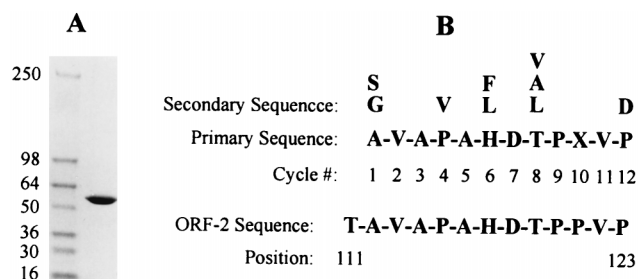


FIG. 3. Purification and N-terminal sequence analysis of the p62-kDa protein. (A) Coomassie blue staining of the p62-kDa protein after Poros HQ/F column purification. The details are described in the Materials and Methods section. (B) Material from the analysis described for panel A was blotted onto a polyvinylidene difluoride membrane and subjected to Edman degradation. The sequencing cycle numbers and the primary sequence derived from the first 12 cycles are indicated. X in cycle number 10 indicates an unidentifiable amino acid. The amino acid sequence from the corresponding region of the published ORF-2 sequence of the Burma strain of HEV is shown at the top, with the number one position being the first amino acid in ORF-2.

ORF-2. Separate studies revealed that there were indeed two possible proteolytic cleavage sites within the last 40 residues of the 62-kDa protein (22, 23).

Construction of a recombinant baculovirus that expresses amino acids 112 to 660 of ORF-2 in Sf9 suspension cultures. The DNA sequence which encodes from Ala-112 to the carboxyl end of ORF-2 was inserted in a baculovirus expression vector, pBlueBacIII. The corresponding recombinant virus vB-BIII-62k was constructed as described in the Materials and Methods section. A methionine codon was incorporated at the 5' end to ensure the proper start of translation. After transfection and four rounds of plaque purification, viral stocks and cell lysates were prepared from 10 individual plaque isolates. Western blot analysis was performed to screen for the viral plaques that could produce a soluble 62-kDa protein (r62-kDa protein) in suspension cultures at the highest level (Fig. 4). Nine of 10 viral isolates produced a soluble 62-kDa doublet similar to that of the p62-kDa protein, indicating that the synthesis of the soluble 62-kDa protein was not dependent on the proteolytic processing. The solubility of the r62-kDa protein ranged from 50 to 100%, depending on the level of expression in each isolate. One isolate was chosen for amplification for further studies.

Comparative immunoreactivities of insect cell-produced ORF-2 proteins. The immunoreactivities of baculovirus-ex-

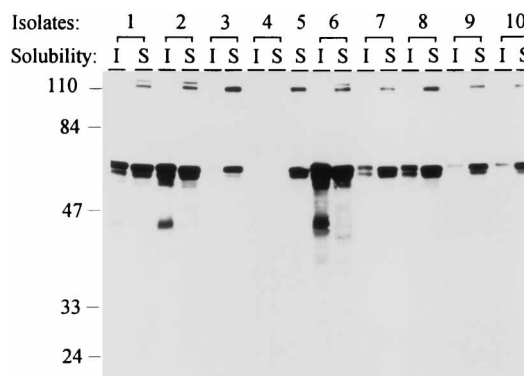


FIG. 4. Expression of the r62-kDa protein in Sf9 suspension cultures. Experimental conditions are essentially the same as those described in the legend to Fig. 1A, except that recombinant virus vBIII-62k was used.

TABLE 1. Comparative ELISA analysis of human HEV-seropositive samples with three antigens, the SG3 antigen and the p62-kDa and the 73-kDa proteins^a

Sample	Anti-HEV response (OD ₄₉₀ ^b) of the following antibodies to the indicated antigen:					
	IgG			IgM		
	SG3 antigen	p62-kDa protein	73-kDa protein	SG3 antigen	p62-kDa protein	73-kDa protein
Borneo S89	0.237	2.527	0.356	0.895	2.579	1.077
MBO283	0.123	0.892	0.147	2.602	2.771	2.558
Sudan 54	0.532	2.652	0.754	2.159	2.717	2.310
Som 055	1.342	2.560	1.283	1.930	2.386	1.539
FVH26	0.077	0.319	0.069	0.379	1.353	0.571
Som 458	0.279	1.525	0.248	0.206	2.441	0.257

^a All three antigens were subjected to freezing and thawing. For details see Materials and Methods section.

^b OD₄₉₀, optical density at 490 nm.

pressed ORF-2 proteins were examined in an ELISA format with HEV-seropositive human serum samples (Tables 1 to 3). An *E. coli*-produced HEV ORF-2 antigen (SG3) containing 332 amino acids of the C terminus of ORF-2 (41) was included for comparison. This protein was previously observed to contain broadly immunoreactive epitopes. In reactions in which equal molar amounts of HEV antigens were coated onto ELISA plates, greater reactivity was seen with the p62-kDa protein than with the SG3 antigen or the 73-kDa protein (Table 1). The limited quantities of the human sera in this panel prevented us from further defining the relative reactivities of each antigen by an endpoint titer assay, but results of this type of assay are presented in Table 3. The 62-kDa protein reacted with low-level antibodies to HEV in some sera that would have gone undetected by using the SG3 antigen (Table 1). The difference in sensitivity between the two antigens was generally more significant in anti-HEV IgM reactions (Table 3). Despite the lower immunoreactivities seen with the 73-kDa protein, we could demonstrate that the freshly purified 73-kDa protein preparations could have increased immunoreactivities sometimes close to that seen with the p62-kDa protein. Freezing and thawing, however, greatly reduced the immunoreactivities of the refolded 73-kDa protein, although the protein remained intact, as examined by Coomassie blue staining. This temperature lability was observed only with the 73-kDa protein.

The r62-kDa protein from the reengineered construct performed nearly identically to the processed one or the p62-kDa protein in detecting HEV IgG (Table 2), suggesting the conformational and immunological similarities of the two proteins.

A small subset of HEV-positive human sera were serially

TABLE 2. Comparison of the immunoreactivities of the r62-kDa, p62-kDa, and the 73-kDa proteins in seropositive samples from regions where HEV is endemic

Sample	Anti-HEV IgG response (OD ₄₉₀ ^a) to the following:		
	r62-kDa protein	p62-kDa protein	73-kDa protein
ABTD80	2.229	2.212	0.344
Mex F387	2.635	2.664	1.069
ABTD 86	2.399	1.689	0.309
MB 2/88	1.961	2.083	0.724

^a OD₄₉₀, optical density at 490 nm.

TABLE 3. ELISA endpoint titers of HEV-seropositive human sera against the SG3 antigen and the r62-kDa and 73-kDa proteins^a

Sample	Anti-HEV titer of the following antibodies to the indicated antigen:					
	IgG			IgM		
	SG3 antigen	r62-kDa protein	73-kDa protein	SG3 antigen	r62-kDa protein	73-kDa protein
F387	1:102,400	1:102,400	1:204,800	<1:100	1:1,600	1:400
KS	1:25,600	1:102,400	1:102,400	1:100	1:800	1:400
AZ	1:3,200	1:51,200	1:3,200	1:400	1:12,800	1:3,200

^a See Materials and Methods section for details.

diluted and assayed to further define sensitivity of the r62-kDa antigen (Table 3). The endpoint ELISA showed that the r62-kDa protein exhibited increased sensitivity, sometimes up to 30-fold, of anti-HEV IgM detection, compared to the sensitivities of detection of currently used HEV antigens such as SG3. This improvement could also be confirmed with sera from experimentally infected primates (data not shown).

DISCUSSION

Our objectives in this study were threefold: (i) to fully characterize the HEV ORF-2 protein species expressed in insect cells, (ii) to identify the most promising candidate in terms of immunoreactivity, and (iii) to design a process to express this candidate protein in a large-scale format for diagnostic assays and vaccine development. Several laboratories have reported expression of antigenic HEV ORF-2 proteins in insect cells. However, results have varied with respect to the size and solubility of recombinant proteins. In the present study, we observed two ORF-2 protein products in Sf9 cells of different origins: the insoluble 73-kDa protein produced in a suspension cell line and the soluble p62-kDa protein from another cell line grown primarily as monolayers. Why was the 73-kDa protein cleaved (in vivo) in one cell line but not in the other is yet to be determined. However, we have determined that (i) the protease(s) for the cleavage of the 73-kDa protein to the 62-kDa protein came from baculovirus rather than host cells and (ii) a switch between suspension and monolayer growth in both cell lines had no effect on the size of the recombinant protein (data not shown). Therefore, the source of cell line is apparently very important for this cleavage.

The ideal recombinant antigen for use in diagnostics and a vaccine should preserve the conformational epitopes of the native protein. Therefore, the solubility of the recombinant protein is an important criterion for antigen selection. The 73-kDa protein is not preferred because of its insolubility and temperature lability. Spontaneous proteolytic cleavage of the 73-kDa protein resulted in a 62-kDa protein that was soluble and stable and that showed improved immunoreactivity. However, reliance on an uncharacterized spontaneous proteolytic cleavage process is not desirable with respect to scale-up and consistency of production of the recombinant protein. In addition, monolayer cultures are not suitable for large-scale recombinant protein production. To overcome these problems, we proposed that the strategy for p62-kDa protein expression be modified.

Recombinant proteins expressed at high levels in both bacterial and insect cells can often become insoluble and accumulate inside the cells in the form of inclusion bodies. It is known that both the primary protein sequence and the rate of protein synthesis are important factors for determining the solubility of recombinant proteins. The conversion from the insoluble 73-

kDa protein to the soluble p62-kDa protein by the proteolytic cleavage suggested that the primary sequence of the p62-kDa protein might be a determining factor for its solubility. Therefore, it was conceivable that this soluble protein could be made by directly expressing the coding sequence of the p62-kDa protein in the insect cultures by using a recombinant baculovirus. This would eliminate the role of proteolytic processing in generating the soluble 62-kDa protein, as indicated in Fig. 1. However, we did have concerns whether (i) modification of the p62-kDa protein sequence by the inclusion of an initiating methionine and (ii) the unique expression of this coding sequence outside the context of the full-length protein would produce a protein with the same desirable physical and immunogenic characteristics shown by the p62-kDa protein. To test this hypothesis, the soluble p62-kDa protein was purified to near homogeneity followed by N-terminal sequencing. The cleavage site that generated the p62-kDa protein from the 73-kDa protein was found between residues Thr-111 and Ala-112 in ORF-2. The truncated ORF-2 was inserted in a baculovirus expression vector to generate the recombinant virus vBBIII-62k. Interestingly, the r62-kDa protein produced on the basis of this expression strategy indeed demonstrated all the properties of the 62-kDa protein that was obtained by cleavage. This approach to resolving the problem of insolubility of recombinant proteins produced in insect cells may be extended to other cases in the future.

There are two explanations for the significant increase in the sensitivity of anti-HEV IgM detection in human serum samples with our newly engineered 62-kDa protein compared to that of antigen SG3 produced by bacteria (1). The 62-kDa protein is longer than the SG3 antigen by 212 amino acid residues and therefore may contain extra epitopes. However, numerous studies performed to map the epitopes in ORF-2 failed to identify any important antigenic determinant within this 212-amino-acid region (5, 6, 13, 14, 40, 41). (ii) Treatment of the insoluble SG3 antigen with the solubilizing agent such as urea during the purification may have destroyed some conformational epitopes that were otherwise preserved in the soluble 62-kDa protein. Interestingly, we had a such experience. In a separate study, we expressed in *E. coli* all but 23 N-terminal amino acids of ORF-2. This 637-amino-acid antigen (ORF-2-sp), which was 88 amino acids longer than the 62-kDa protein, showed much weaker immunoreactivities than the 62-kDa protein (data not shown). This seems to support our second hypothesis of the contribution of a conformational epitope(s) in the undenatured soluble protein to the improved immunoreactivity.

In several studies in which more human sera were assayed, we found that the specificity of the r62-kDa protein was not compromised, despite the increased sensitivities of anti-HEV antibody detection (data not shown).

The soluble r62-kDa protein should be suitable for the development of an anti-HEV IgM assay as well as for HEV vaccine development. Indeed, preclinical testing indicated that the r62-kDa antigen was capable of eliciting protective immunity in cynomolgus macaques against a very stringent heterologous challenge with 1,000 to 10,000 cynomolgus monkey 50% infectious doses of the Mexico isolate of HEV (9, 39).

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