Expression of Hepatitis E Virus Putative Structural Proteins in Recombinant Vaccinia Viruses

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Hepatitis E virus (HEV) is a polyadenylated, positive-stranded RNA virus which is a major cause of enterically transmitted non-A, non-B hepatitis in many developing countries. The viral genome contains three different open reading frames (ORFs): ORF1, which is believed to encode nonstructural proteins, and ORF2 and ORF3, which are believed to encode structural proteins. The full-length putative structural proteins encoded by ORF2 and ORF3 of HEV have been cloned and expressed in recombinant vaccinia virus. Proteins encoded by ORF2 and ORF3 when expressed in vaccinia virus are recognized by pooled sera obtained from individuals with acute hepatitis E. Vaccinia-expressed viral gene products of HEV will have utility in characterizing the cell-mediated immune response to HEV.

Enteroically transmitted non-A, non-B hepatitis occurs in epidemics or sporadically in many geographic locations, including India (13, 14, 27, 32), Nepal (10), Burma (14, 26), Pakistan (3), Mexico (30), the former Soviet Union (1), Africa (2, 24), North America (4), and Western Europe (17). Acute outbreaks of enterically transmitted non-A, non-B hepatitis primarily affect young to middle-aged adults and are often associated with a high mortality rate in infected pregnant women, approaching 20% in many reported epidemics (20). Recently, an enterically transmitted non-A, non-B hepatitis agent has been cloned and sequenced and has been termed hepatitis E virus (HEV) (22, 29). Hepatitis E disease is transient and self-resolving and is similar to hepatitis A clinically and epidemiologically except for the high mortality rate in pregnant women. The virus appears to be a polyadenylated, positive-stranded RNA virus with three apparent open reading frames (ORFs). The largest, ORF1, is believed to encode nonstructural proteins such as the RNA-dependent RNA polymerase (22), a putative helicase (5), and other nonstructural proteins recognized from other consensus motifs (15). The second major ORF (ORF2) and the small third ORF (ORF3) have been shown to encode highly immunogenic antigens and may encode the structural proteins of HEV (29, 33). In this study, we report on the expression of the full-length HEV proteins encoded by ORF2 and ORF3 produced by recombinant vaccinia viruses.

The plasmid BET 6-1 (28, 29), which contains the entire HEV (Burma strain) ORF3, and a recombinant λgt10 bacteriophage (BET1) containing all of ORF2 except for 19 nucleotides at the 5′ end (28) were used as templates in the PCR (23) which resulted in amplified DNA fragments of the entire ORF3 and ORF2 with flanking SmaI restriction endonuclease cleavage sites. For the ORF2 fragment, 35 PCR cycles were performed in a 100-μl volume containing 0.1 μM of each of the two primers, PCR buffer (United States Biochemicals, Cleveland, Ohio) (1.5 mM MgCl₂), and 1 U of Taq polymerase (United States Biochemicals). Each cycle consisted of a denaturation at 95°C for 45 s, primer annealing for 2 min at 55°C, and primer extension at 72°C for 2 min. Primers used to amplify ORF2 were primer 1, 5′-GCG-CGC-CCC-GGG-ATG-CGC-CCT-CGG-CCT-ATT-CTT-TGT-TTG-CGT-CTC-ATG-TTT-TGG-CTC-ATG-GTG-TTG-GTG-CTC-CCT-CGG-CCT-ATG-TCT-TGG-CTC-CTC-ATG-CCT-CGT-CTC-AGT-ACC-CAC-CTT-CAT-CTT-AAG-GCG-CTG-AAG-GTC-AGC-GAC-AGT-3′, corresponding to 54 bases from nucleotide 5147 to 5200, and primer 2, 5′-GCG-CGC-CCC-GGG-CTA-CTA-CAA-CTC-CTC-GCG-AGT-TTT-ACC-CAC-CTT-CAT-CTT-AAG-GCG-CTG-AAG-GTC-AGC-GAC-AGT-3′, corresponding to 57 bases from 7072 to 7129 (29).

The ORF3 fragment was amplified in a 100-μl volume containing 0.1 μM of each primer, PCR buffer, 2 mM MgSO₄, 1 U of Vent DNA polymerase (New England Biolabs, Beverly, Mass.). Each cycle consisted of denaturation at 95°C for 45 s, primer annealing for 2 min at 45°C, and primer extension at 72°C for 2 min. Primer 3, 5′-GCG-CGC-CCC-GGG-ATG-AAT-AAC-ATG-TCT-TTT-GCT-GCG-CCC-3′, corresponding to 27 bases from 5105 to 5132, and primer 4, 5′-GCG-CGC-CCC-GGG-TTA-TTA-GCG-GCG-CGG-CAG-CTG-TGG-GG-GCG-CCC-CAG-TGG-3′, which corresponds to 24 bases from 5454 to 5478 (25), were used to amplify ORF3. Each primer also contained flanking SmaI restriction endonuclease cleavage sites (underlined in the sequences above) and six additional nucleotides at the 5′ end. The ORF2 PCR product was digested with XmaI (New England Biolabs) and ligated into the compatible XmaI site in transfer vector pSC65 (a gift of Sekhar Chakrabarti, National Institute of Cholera and Enteric Diseases, Calcutta, India) which was previously digested with XmaI and treated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, Ind.). The ORF3 PCR product was digested with SmalI (New England Biolabs) and ligated into the compatible SmalI site in transfer vector pSC65 which was previously digested with SmalI and treated with alkaline phosphatase (Boehringer Mannheim). After transformation of the ORF2-containing plasmid into INVVα competent cells and the
ORF3-containing plasmid into DH5α, ampicillin-resistant colonies were screened for the presence of the respective recombinant plasmids (23). The orientation of the inserted fragments in pSC65 was determined by restriction enzyme analysis by digesting possible ORF2-containing plasmids with KpnI and possible ORF3-containing plasmids with XmaIII and KpnI (Bethesda Research Laboratories, Gaithersburg, Md.).

Correct orientations of the 1,988-bp ORF2 fragment and the 378-bp ORF3 fragment in the transfer vector were confirmed by restriction enzyme analysis. pMK1 (Fig. 1), a recombinant plasmid containing the ORF2 fragment, was digested with KpnI, and the correct orientation of the fragment within the plasmid was confirmed by the presence of a 1,671-bp fragment and a fragment of approximately 7,388 bp (data not shown). Similarly, pMK2 (Fig. 1), a recombinant plasmid containing the ORF3 fragment, was digested with KpnI and XmaIII and the correct orientation of the fragment within the plasmid was confirmed by the presence of a 247-bp fragment and a fragment of approximately 7,202 bp (data not shown).

The ORFs were inserted downstream of the synthetic hybrid early/late vaccinia virus promoter of transfer plasmid pSC65. This vector allows both dominant selection and β-galactosidase screening of recombinant vaccinia virus. The transfer plasmids, which contain flanking vaccinia virus thymidine kinase sequences, were introduced into vaccinia virus by using a modification of the standard homologous recombination method (16). Briefly, the CV-1 cell line (ATCC CCL 70) was infected by wild-type vaccinia virus (strain WR) followed by calcium chloride-mediated transfection of the transfer plasmids into the vaccinia virus-infected cells. After 24 h of incubation, the infected cells were mechanically lysed and the released virus was used to infect TK−143B cells (ATCC CRL 8303) in the presence of 5-bromodeoxyuridine. After 48 h of infection, the infected cells were overlaid with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the individual blue plaques were picked. Individual plaques underwent three plaque purification procedures before being amplified in TK− cells under 5-bromodeoxyuridine selection. Monolayer cultures of TK−143B were infected with ORF2- or ORF3-containing recombinant vaccinia virus or with parental vaccinia virus WR strain.

After harvesting, the cells were centrifuged at 3,000 × g for 10 min and aliquots of the cell pellets formed were resuspended in 2× protein sample buffer and boiled for 10 min before equal volumes of each protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide).

Proteins separated by SDS-PAGE were electroblotted (23) onto nitrocellulose membranes in transfer buffer containing 25 mM Tris, 190 mM glycine, and 20% methanol. Membranes were blocked with phosphate-buffered saline (PBS) containing 1% sodium caseinate, 0.05% sodium azide, and 0.05% Tween 20 for 1 h at room temperature. The nitrocellulose membranes were then incubated for 18 h at 4°C with a 1:200 dilution of pooled serum obtained from patients acutely infected with HEV (9). After being washed four times with PBS containing 0.05% Tween and 0.05% sodium azide (wash buffer), the membranes were incubated with a 1:1,000 dilution of alkaline phosphatase-labeled goat anti-human immunoglobulin G and immunoglobulin M (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) for 1 h at room temperature. After additional washing, the nitrocellulose membranes were incubated for 15 min with nitroblue tetrazolium (0.1 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (0.2 mg/ml) in diethanolamine buffer (Kirkegaard & Perry).

The recombinant vaccinia virus-derived ORF2 protein (with a calculated molecular mass of approximately 70.9 kDa) and the recombinant vaccinia virus-derived ORF3 protein (with a calculated molecular mass of 12.7 kDa) were observed in the respective cell pellets (Fig. 2). Both recombinant proteins were identical in size to the same recombinant proteins expressed in baculovirus (data not shown [7]).

In the present study, we have chosen to express the putative structural proteins of HEV in vaccinia virus. The advantages of using vaccinia virus for expression of HEV proteins are numerous. Vaccinia virus features a series of favorable properties, the most important of which are a broad host range and a high efficiency of infection, that make them uniquely suited as flexible expression vectors (11). Furthermore, recombinant vaccinia virus has proved invaluable for synthesizing biologically active proteins and for determining the targets of humoral and cell-mediated immunity to these foreign proteins (18). These features are extremely important in studying HEV, since sufficient quantities of HEV are not readily available for structural or functional analysis despite the recent description of limited growth of HEV in a cell culture system (8).
Cloned PCR products used in the present study to generate the recombinant vaccinia virus-expressed ORF2 and ORF3 proteins were derived from the HEV Burma strain (22). Since there appears to be some divergence in the nucleotide sequences of HEV isolates from different geographical regions (21, 31), it is not clear whether antibodies generated against the structural proteins of a single HEV strain will recognize all HEV strains. In addition, there is no clear evidence at present that either of these two recombinant virus strains are effective in inducing immunity to HEV, although antibody responses against these two proteins have been previously described (7, 12).

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