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# Expression of the hepatitis E virus ORF1

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**Summary.** Hepatitis E virus (HEV) is an unclassified, plus-strand RNA virus whose genome contains three open reading frames (ORFs). ORF1, the 5′ proximal ORF of HEV, encodes nonstructural proteins involved in RNA replication which share homology with the products of the corresponding ORF of members of the alphavirus-like superfamily of plus-strand RNA viruses. Among animal virus members of this superfamily (the alphavirus and rubivirus genera of the family *Togaviridae*), the product of this ORF is a nonstructural polyprotein (NSP) that is cleaved by a papain-like cysteine protease (PCP) within the NSP. To determine if the NSP of HEV is similarly processed, ORF1 was introduced into a plasmid vector which allowed for expression both in vitro using a coupled transcription/translation system and in vivo using a vaccinia virus-driven transient expression system. A recombinant vaccinia virus expressing ORF1 was also constructed. Both in vitro and in vivo expression under standard conditions yielded only the full-length 185 kDa polyprotein. Addition of co-factors in vitro, such as divalent cations and microsomes which have been shown to activate other viral proteases, failed to change this expression pattern. However, in vivo following extended incubations (24–36 hours), two potential processing products of 107 kDa and 78 kDa were observed. N- and C-terminus-specific immunoprecipitation and deletion mutagenesis were used to determine that the order of these products within the NSP is $NH_2$-78 kDa-107 kDa-COOH. However, site-specific

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mutagenesis of $Cys_{483}$, predicted by computer alignment to be one member of the catalytic dyad of a PCP within the NSP, failed to abolish this cleavage. Additionally, sequence alignment across HEV strains revealed that the other member of the proposed catalytic dyad of this PCP, $His_{590}$, was not conserved. Thus, the cleavage of the NSP observed following prolonged in vivo expression was not mediated by this protease and it is doubtful that a functional PCP exists within the NSP. Attempts to detect NSP expression and processing in HEV-infected primary monkey hepatocytes were not successful and therefore this proteolytic cleavage could not be authenticated. Overall, the results of this study indicate that either the HEV NSP is not processed or that it is cleaved at one site by a virally-encoded protease novel among alpha-like superfamily viruses or a cellular protease.

## Introduction

Hepatitis E virus (HEV) is the causative agent of enterically transmitted non-A, non-B hepatitis, which, unlike other forms of viral hepatitis, is associated with a high mortality rate (10–20%) in pregnant women [13, 14, 29]. HEV is a small, spherical, nonenveloped virus, approximately 27–32 nm in diameter, which has a single-stranded, positive-sense RNA genome of approximately 7.5 kilobases that contains three partially overlapping open reading frames (ORFs) [2, 3, 4, 27, 35]. ORF1 (nt 28 to 5107) encodes for nonstructural proteins which include the viral replicase. ORF2 (nt 5147 to 7126) encodes the structural protein and ends 68 nts from the poly A tract. ORF3 (nt 5106 to 5474) overlaps ORF1 by one nt and ORF2 by 328 nts and encodes a small phosphoprotein which associates with the cytoskeleton [37].

Based on virion morphology and genome organization, HEV was originally classified as a member of the family *Caliciviridae* [6]. However, computer-assisted alignment of the amino acid sequence of the HEV ORF1 with corresponding ORFs of other viruses found no homology with caliciviruses or other picorna-like viruses but did reveal limited but significant homology with members of the alphavirus-like superfamily of RNA viruses [26], particularly rubella virus (RUB), a Togavirus, and beet necrotic yellow vein virus (BNYVV), a Furovirus. Viruses belonging to the alphavirus-like superfamily have genomic RNAs with 5′ cap structures, 3′ poly As, and produce subgenomic mRNAs while picorna-like viruses have 5′-terminal VPgs and 3′ poly As and may or may not produce subgenomic RNAs. Therefore, HEV was recently removed from the family *Caliciviridae* family and is currently unclassified [25].

Computer analysis also revealed a number of putative amino acid motifs within HEV ORF1 including, from N to C terminal: 1) a methyltransferase (MT); 2) a domain of unknown function called Y; 3) a papain-like cysteine protease (PCP); 4) a proline-rich “hinge” domain; 5) a domain X of unknown function; 6) an NTP-binding sequence associated with helicase activity; and 7) an RNA-dependent RNA polymerase [16]. Most of these motifs are found in the corresponding ORFs of alphavirus-like superfamily members and all of them are found in the corresponding ORFs of RUB and BNYVV (these ORFs are gener-

ally termed nonstructural protein ORFs or NSP-ORFs). The presence of an MT motif suggests that the HEV RNAs are capped. The PCP is characteristic of the alphaviruses, RUB and BNYVV, and PCPs are found in members of other plus strand RNA virus families, however, the amino acid alignment between the putative HEV protease and these other viral PCPs was weak [16]. While the function of the conserved X domain is unknown, it flanks a PCP domain in the polyproteins of several plus strand RNA viruses.

The processing of individual proteins from polyprotein precursors by specific, limited proteolysis is common to the genome expression strategy of all plus strand RNA viruses of animals (reviewed in [17, 36]). BNYVV and RUB both use such a strategy: RNA1 of BNYVV contains a single ORF which encodes for a 220 kDa nonstructural polyprotein that is cleaved by a virally encoded PCP into a 150 kDa N-terminal product and a 66 kDa C-terminal product [11]. Similarly, the RUB NSP-ORF encodes a 240 kDa nonstructural polyprotein (2205 aa) which is cleaved between the $Gly_{1301}$ and $Gly_{1302}$ by a PCP within the polyprotein to yield a 150 kDa N-terminal product and 90 kDa C-terminal product [22].

The goal of this study was to investigate the potential processing of the HEV ORF1 nonstructural protein product. To date, only limited success has been obtained towards inducing HEV to replicate in cell culture [12, 33], and in such systems virus-specific macromolecular synthesis required extremely sensitive methods for detection. Therefore, a cDNA containing HEV ORF1 was introduced into plasmid vectors from which both in vitro and in vivo expression could be conducted. With the results of the expression study in hand, we also attempted to detect ORF1 expression in HEV-infected hepatocytes in culture.

## Materials and methods

### *Recombinant DNA manipulations*

Cloning procedures were as described in Maniatis et al. [21]. *Escherichia coli* XL-1 Blue was used as the host strain for plasmid cloning and amplification. Transformation was done by electroporation [8]. Plasmid digestions with restriction enzymes (New England BioLabs, Beverly, MA) were carried out using the supplier's protocols and buffers. T4 DNA ligase was purchased from Promega (Madison, WI). The relevant regions of all constructs and all mutations were confirmed by sequencing to ensure that the desired modifications had been made.

### *Constructs*

#### pBSORF1 and 625DNA

pBSORF1 was assembled from cDNA clones used in the original sequence determination of the HEV genome (Burmese strain) [32] and contained nt 1 to 5679 of the genome inserted into a pBluescript II SK + plasmid vector (Stratagene, La Jolla, CA) with the ORF1 sequences under control of the T7 RNA polymerase promoter. 625DNA was a second independently generated cDNA clone of ORF1 from the Bumese strain that contained nt 27 to 7194 of the genome assembled in a pSP71 vector with the ORF1 sequences under control of the T7 promoter. Amino acid differences in ORF1 in the 625DNA construct in comparison to the originally reported Burmese strain sequence [32] were a threonine at residue 1077 in

place of the glycine (T1077G), C1088W, A1132P, A1254V, R1315G, and L1543F. A stop codon at aa 437 was changed to a consensus tryptophan to generate a complete open reading frame.

### pMAL constructs

pBSORF1 was digested by *Xho*I and *Hin*dIII to generate a 5679 bp fragment that was then ligated into the *Sal*I/*Hin*dIII site of the pMAL-c2 expression vector (New England Biolabs, Beverly, MA). In this construct, pMAL/ORF1, the HEV ORF1 was inserted in frame downstream from the *malE* gene of *E. coli.* As shown in Fig. 1, the pMAL/ORF1 C-terminal construct (pMAL/ORF1C) construct was generated by digesting pMAL/ORF1 with *Bam*HI and religating. This construct contained nt 2959 to 5108 of the HEV genome which, when expressed, was expected to produce a 122 kDa (43 + 79 kDa) MBP fusion protein containing the last 716 aa of the NSP. The pMAL/ORF1 N-terminal construct (pMAL/ORF1N) was generated by digesting pMAL/ORF1 with *Nru*I and *Pml*I (both blunt end restriction enzymes) and religating in the presence of the blunt end linker with sequence CTAGCTAGCTAG to introduce a stop codon (TAG) in all three reading frames and an *Nhe*I restriction site for verification. This construct contained nt 28 to 2429 of the HEV genome which, when expressed, was expected to produce a 131 kDa (43 + 88 kDa) MBP fusion protein containing the first 800 aa of the NSP.

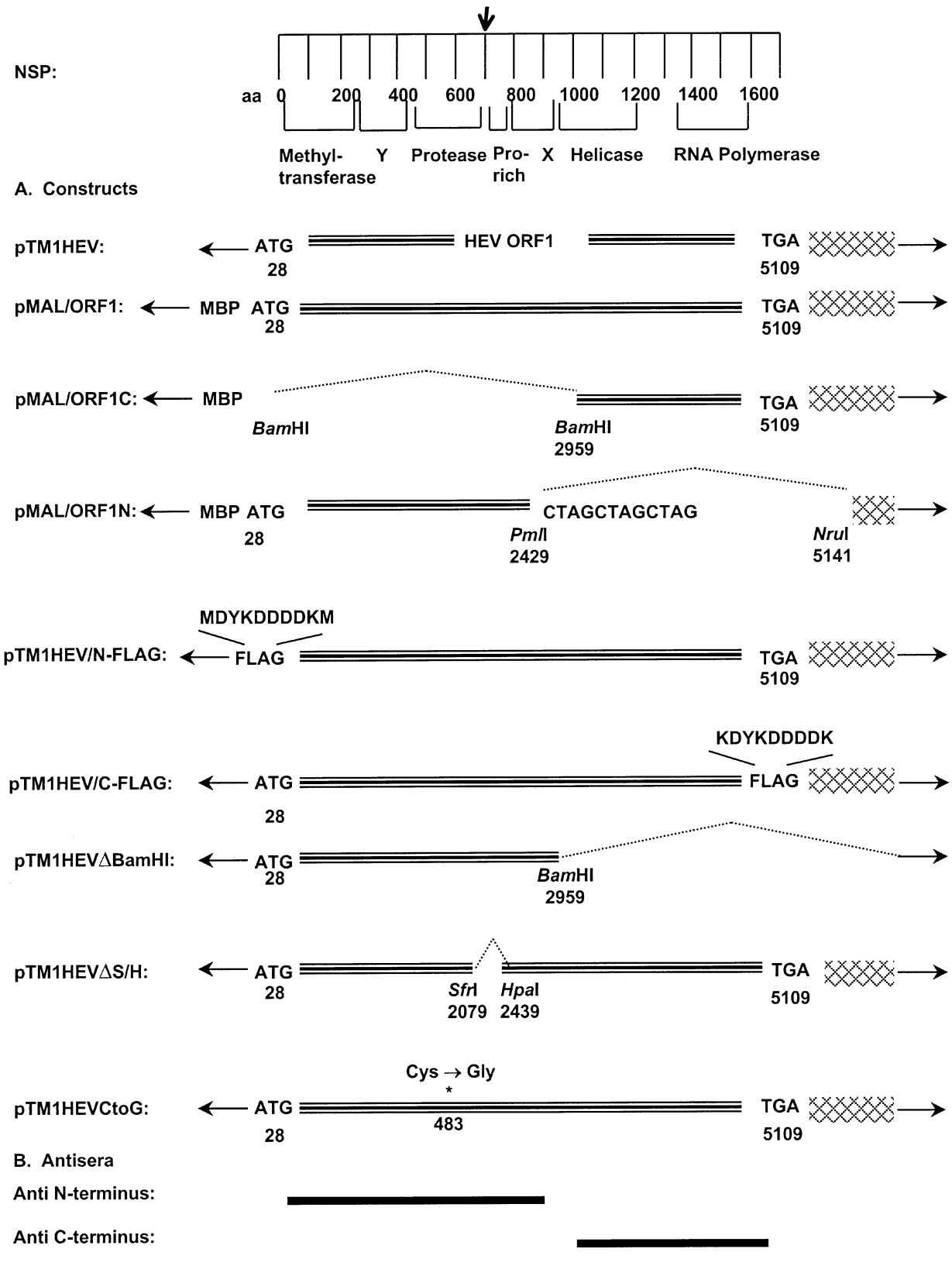
### pTM1HEV and vvHEV

pTM1, a mammalian expression vector derived from a pUC plasmid and designed for gene expression under the control of the T7 promoter vector, was kindly provided by by B. Moss [23]. In this vector, the T7 transcripts begin with the EMCV IRES element for efficient, cap independent initiation of translation. A 5651 bp fragment of the HEV genome from pBSORF1 containing nt 28 to 5679 that included the entire HEV ORF1 (nt 28 to 5109) was introduced into pTM1 using the *Nco*I site at nt 28 of the HEV genome to align the authentic AUG start of HEV with the AUG used by the IRES. The pTM1 vector also contains the vaccinia virus TK gene flanking sequences for uptake of the expressed gene into vaccinia virus by homologous recombination and selection for TK-mutants. Such a recombinant, vvHEV, was generated using pTM1HEV and the vvWT Western Reserve strain by standard methodology [20].

### FLAG constructs

The FLAG octapeptide (DYKDDDDK) was introduced at the N- or C-terminus of the NSP expressed from pTM1HEV by PCR (Fig. 1). The N-terminal FLAG was introduced using a forward primer (5′-CATGCCATGGACTATAAGGACGACGACGACAAGATGGAGGCCCATCAGTTTATT-3′) which contained an *Nco*I site, a start codon, the 8 codons

**Fig. 1.** Constructs used in this study. pTM1HEV, pMAL/ORF1, pMAL/ORF1N, pMAL/-ORF1C, pTM1HEV/N-FLAG, TM1HEV/C-FLAG, pTM1HEV$\Delta$*Bam*HI, pTM1- HEV-$\Delta$S/H, and pTM1HEVCtoG are shown in **A**. pTM1HEV was used to construct vvHEV. Vector sequences = ⟷; HEV ORF1 sequence = ═; HEV genome sequence outside of ORF1= ▨. The NSP encoded by ORF1 (scale in amino acids), including computer-predicted motifs, is shown at the top of the diagram for reference. The arrow indicates the region containing a cleavage site that would produce the 78 kDa and 107 kDa products observed in vivo. In **B** are shown regions of the HEV NSP recognized by the N- and C-terminal-specific antisera raised against the pMAL/ORF1N and -C fusion products, respectively


NSP:
aa 0 200 400 600 800 1000 1200 1400 1600
Methyl-transferase
Y
Protease
Pro-rich
X
Helicase
RNA Polymerase
A. Constructs
pTM1HEV:
ATG 28
HEV ORF1
TGA 5109
pMAL/ORF1:
MBP
ATG 28
TGA 5109
pMAL/ORF1C:
MBP
BamHI
BamHI 2959
TGA 5109
pMAL/ORF1N:
MBP ATG 28
CTAGCTAGCTAG
PmlI 2429
NruI 5141
MDYKDDDDKM
pTM1HEV/N-FLAG:
FLAG
TGA 5109
KDYKDDDDK
pTM1HEV/C-FLAG:
ATG 28
FLAG
pTM1HEVΔBamHI:
ATG 28
BamHI 2959
pTM1HEVΔS/H:
ATG 28
SfrI 2079
HpaI 2439
TGA 5109
Cys → Gly
*
pTM1HEVCtoG:
ATG 28
483
TGA 5109
B. Antisera
Anti N-terminus:
Anti C-terminus:

encoding the FLAG peptide, and the first 30 nt of ORF1. The C-terminal FLAG was introduced by using a reverse primer (5′-AGTTAGAACACAGCCCACCCTCTGATATTCCTG-CTGCTGCTGTTCACTCTTAAGCCG-3′) which contained an *Eco*RI site, a stop codon, the 8 codons encoding the FLAG peptide, and the last 30 nt of the HEV ORF1.

### Deletion constructs

pTM1HEVΔ*Bam*HI was generated by digesting pTM1HEV with *Bam*HI and religating, resulting in a deletion of the last 2141 nt of the HEV ORF1. pTM1HEVΔ*Bam*HI contained nt 28 to 2959 of the HEV ORF1 as shown in Fig. 1. pTM1HEVΔS/H was generated by double digesting pTM1HEV with *Srf* I and *Hpa*I and religating producing an in-frame deletion between nts 2079 through 2439 of the HEV genome as illustrated in Fig. 1.

### pTM1HEV/CtoG

Mutagenesis of the putative catalytic $Cys_{483}$ to Gly was accomplished by PCR using a primer (5′-CATTATCATGACCCTGGTCGCCGACGGCGCCTTCTGCAGCTGAAGGAAC**C**GG-TGCACTCCTGAC-3′) which contained a single change in the ORF1 sequence corresponding to nucleotide position 1475. The mutation changed a **T**GC codon to a **G**GC codon.

### *Expression and preparation of MBP fusion proteins*

Synthesis of MBP fusion proteins was induced in *E. coli* XL-1 Blue cells harboring the recombinant pMAL plasmids with isopropylthiogalactoside (IPTG) at a final concentration of 0.3 mM. The large MBP fusion proteins were isolated as inclusion bodies and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gel slices containing the fusion proteins were emulsified in PBS for use as immunogens in rabbits. Immunizations were done by Cocalico Biologicals, Inc. (Reamstown, PA); two rabbits were immunized with each fusion protein on the following schedule: The initial injection was with 100 μg of protein in complete Freund's adjuvant per rabbit. Boosts with 50 μg of protein in incomplete Freund's adjuvant were done at 14 and 21 days after the initial injection. The routes of injection were subcutaneous and intramuscular at multiple sites. Test bleeds were taken at 30 and 60 day postinoculation. Productive bleeds were performed at 90 days postinoculation.

### *In vitro coupled transcription-translation*

A coupled transcription-translation system (TNT; Promega, Madison, WI) based on bacteriophage T7 RNA polymerase and rabbit reticulocyte lysates was used for in vitro synthesis of proteins from plasmid templates according to the manufacturer's protocols in the presence of [$^{35}$S] Translabel (ICN, Costa Mesa, CA). Plasmid templates were purified by anion-exchange chromatography on QIAGEN columns (Qiagen,Dusseldorf).

### *In vivo expression*

HeLa (human epitheloid carcinoma), Vero (monkey kidney), Hep-G2 (human hepatocellular carcinoma), and Chang Liver (human liver) cell lines (ATCC; Rockville, MD) were grown at 35 °C under 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL) supplemented with 20 μg/ml gentamicin and 8% fetal bovine serum (FBS). The recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase was generously provided by B. Moss and has been previously described [23].

Monolayers of confluent cells in 35-mm$^2$ tissue culture dishes were co-infected with vvHEV and vTF7-3, each at an m.o.i. of 5 pfu/cell. For transfection, monolayers of ∼70%

confluent cells in 35-mm$^2$ tissue culture dishes were infected with vTF7-3 at an m.o.i. of 5 pfu/cell. Following adsorption for 1–2 h, the cells were washed and the medium was replaced with D-MEM containing no serum. Two to 24 h postinfection (or when CPE was visible), infected cells were transfected with plasmid DNA by using Lipofectin (GIBCO-BRL) according to the manufacturer's protocols. For each 35 mm$^2$ culture dish, 10 μg of DNA was diluted in 250 μl of serum-free DMEM and 15 μl of Lipofectin was diluted in a separate 250 μl aliquot of serum-free D-MEM. The two dilutions were mixed together, incubated at room temperature for 15 min, and then applied to the cells for 2 to 24 h at 37 °C. Following coinfection/transfection, cells were washed with methionine-free DMEM containing 2% dialyzed FBS and radiolabeled with methionine-free DMEM containing $^{35}$S Trans label (ICN, 1177 Ci/mmol; 11.88 mCi/ml) at a concentration of 25 μCi/ml for 2 to 24 h. At the end of the labeling period, the cells were washed three times with PBS and lysed in 400 μl of RIP buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 3 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing the Complete protease inhibitor cocktail (Boehringer-Mannheim). Cell debris was removed by centrifugation and the supernatant fluids were stored at −70 °C until use.

### *Immunoprecipitation*

Prior to immunoprecipitation, SDS and 2-mercaptoethanol were added to concentrations of 3% and 1% respectively, samples were boiled for 3 min and then clarified at 10,000 for 10 min. The supernatants were then diluted 1:10 in RIP buffer without SDS containing the Complete protease inhibitor cocktail (Boehringer-Mannheim) and incubated with 50 μl of 10% (wt/vol) Protein A-Sepharose CL-4B (Pharmacia) for 1 h at room temperature to preclear the lysate. Following a 10 min centrifugation, the supernatant was removed and incubated with 4 μl of anti-fusion protein serum or 1 μl of the anti-FLAG mAb (IBI-A Kodak, New Haven CT) overnight at 4 °C. 100 μl of 10% (wt/vol) Protein A-Sepharose CL-4B was then added and incubation was continued for 2 h at room temperature. Immunoprecipitation complexes were collected by centrifugation, washed three times with RIP wash buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 3 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), and resuspended in 50 μl of gel sample buffer. Following dissociation at 95 °C for 3 min, the Sepharose beads were pelleted by centrifugation, and 30 μl of supernatant was loaded per lane on an SDS-PAGE gel.

### *SDS-PAGE*

Protein samples to be resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were mixed 1:1 with sample buffer (1.25% SDS, 12.5% glycerol, 1.25% 2-mercaptoethanol, and 0.0025% bromophenol blue in 0.0625 M Tris-HCl, pH6.8) and boiled for 3 min before application to the gel. SDS-PAGE was conducted using the procedure of Laemmli [18], with 5% acrylamide stacking gels and 8, 10, 12 or 15% acrylamide resolving gels. The acrylamide-bisacrylamide ratio was 37.5:1. Gels containing isotopically labeled proteins were dried and exposed to X-ray-film.

### *Radiolabeled primary hepatocyte lysates*

Lysates of radiolabeled uninfected and HEV-infected primary hepatocytes from cynomolgous macaques were prepared as previously described [33]. Seven or 14 days post-infection, cells in a 30 mm$^2$ culture dish were radiolabeled overnight and then lysed in RIP buffer.

## Results

For these studies, two parental Burmese strain HEV ORF1 clones were employed: pBSORF1 which was constructed from cDNAs used in the original genomic se-

quence determination [32] and which contained nts 1–5679 of the genome, and 625DNA, an independently derived Burmese strain construct that contained nts 27–7194 of the genome. Both of these parental constructs were in vectors with T7 RNA polymerase promoters upstream from the ORF1 sequences and in both cases, the resulting transcript initiated with a short leader consisting of a combination of vector and HEV sequences preceeding ORF1. To optimize translation, the ORF1 sequences from pBSORF1 were introduced into pTM1 [23], a vector in which the T7 RNA polymerase promoter is followed downstream by the encephalomyocarditis virus internal ribosome entry site (IRES) for cap-independent initiation of translation. In this construct, termed pTM1HEV (Fig. 1A), the initiating AUG of ORF1 was aligned with the AUG utilized by the IRES element. pTM1 also contains the vaccinia virus TK gene flanking sequences to facilitate construction of recombinant vaccinia viruses by homologous recombination; a vaccinia virus expressing the HEV ORF1, vvHEV, was constructed. The pTM1 construct and its derivatives described below were used in a coupled transcription/translation system for in vitro expression or transfected into cells infected with a recombinant vaccinia virus, VT7F-3, expressing the T7 RNA polymerase for in vivo expression. Similarly, to study in vivo expression, cells were co-infected with vvHEV and VT7F-3.

N- and C-terminal halves of the ORF1 were also inserted into the pMAL vector to generate constructs which expressed the N- or C-terminal halves of the HEV NSP as maltose-binding fusion proteins (Fig. 1A). These fusion proteins were purified for use as immunogens in rabbits to generate N- and C-terminal-specific antisera. The N-terminal-specific antisera should recognize epitopes within the first 800 aa of the HEV NSP while the resulting C-terminal-specific antisera should recognize epitopes within the last 716 aa of the HEV NSP (Fig. 1B).

To assist in the identification of the N- and C-terminal processing products, two additional constructs, pTM1HEV/N-FLAG and pTM1HEV/C-FLAG were generated which contained the FLAG peptide (DYKDDDDK) at the N- or C-terminus of the NSP respectively (Fig. 1A). Products of these constructs can be immunoprecipitated with commercially available anti-FLAG monoclonal antibodies.

### *In vitro expression*

Since many viral proteases have been successfully investigated in vitro, HEV ORF1 was initially expressed using a coupled in vitro transcription/translation system. Both parental plasmids, pBSORF1 and 625DNA, and the pBSORF1 derivative, pTM1HEV, gave similar results; the expression of pTM1HEV over a 90 min time course is shown in Fig. 2A. A 185 k Da product, the predicted size of the ORF1 primary translation product, was present by 40 minutes, however other significant products were not observed. As shown in Fig. 2B, the 185 kDa products translated from pTM1/N-FLAG and /C-FLAG were specifically immunoprecipitated by an anti-FLAG monoclonal antibody, confirming that this species is the primary translation product of ORF1. There were a number of faint, lower MW bands produced by all three constructs. These were immunoprecipitated by the

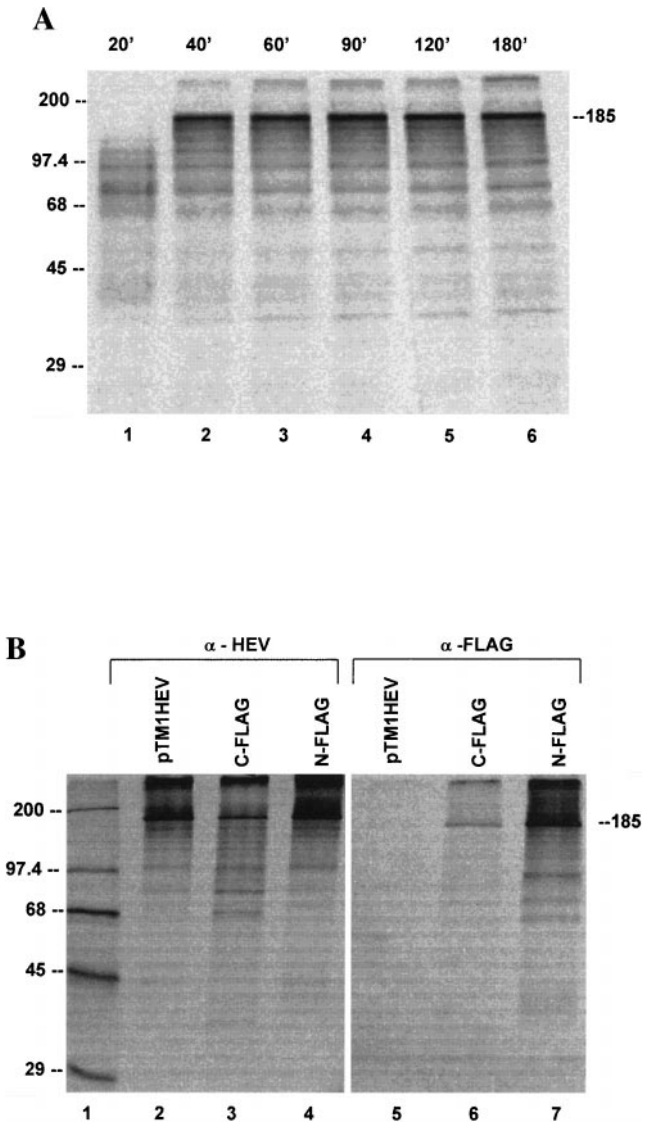



**Fig. 2.** In vitro expression of pTM1HEV. **A** Timecourse. Transcription/translation reaction mixtures programmed with pTM1HEV were incubated at 30 °C. Individual reactions were stopped at 20, 40, 60, 90, 120, and 180 min (*1–6*, respectively). **B** Immunoprecipitation. Transcription/translation reaction mixtures programmed with pTM1HEV (*2, 5*), pTM1HEV/C-FLAG (*3, 6*), or pTMA1HEV/N-FLAG (*4, 7*) were incubated at 30 °C for 3 h and either immunoprecipitated with HEV-specific antisera (N- and C-terminal sera combined; *2–4*) or the anti-FLAG Mab (*5–7*). In both panels, the radiolabeled polypeptides produced were analyzed by SDS-PAGE using a 10% gel. The migrations of the MW standards are shown in the left margin (the standards are in *1* of **B**) and the full-length 185 kDa NSP is shown in the right margin

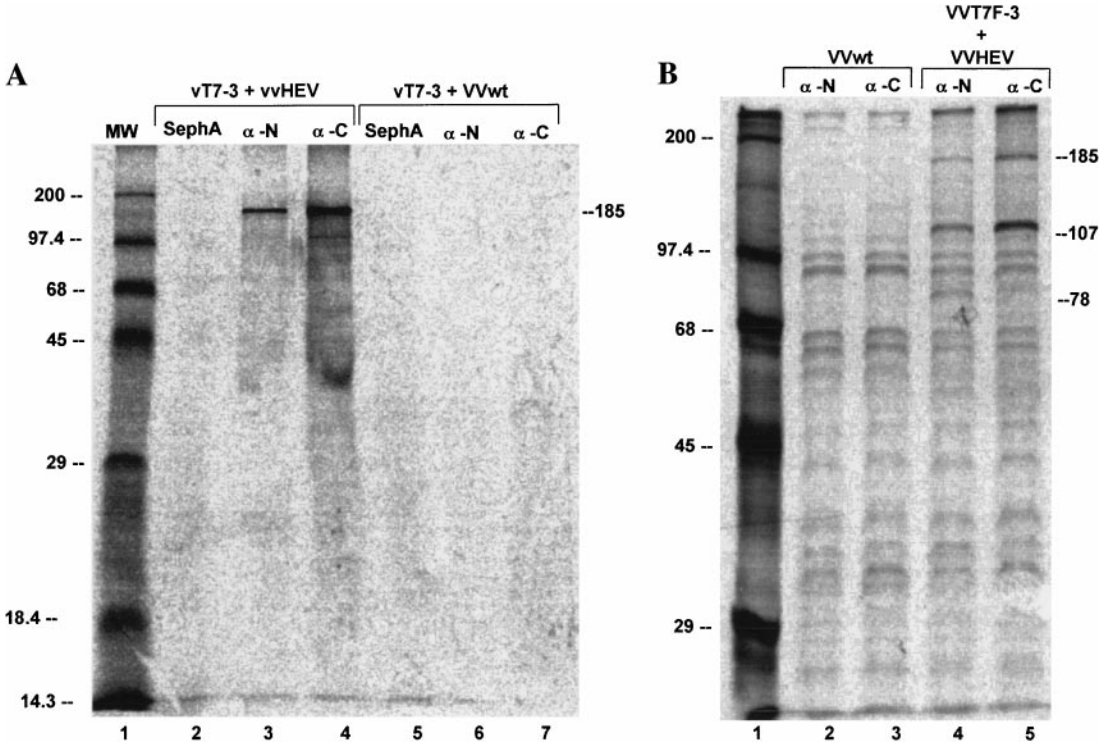



**Fig. 3.** In vivo expression by vvHEV. Monolayers of Chang Liver cells were infected with vvWT alone (**A**, *5–7*; **B**, *2–3*) or vvHEV plus vTF7-3 (**A**, *2–4*; **B**, *4–5*). In **A**, at 2 h post-infection the cells were radiolabeled for three hours. In **B**, at 12 h post-infection cells were radiolabeled for 24 h. Lysates were immunoprecipitated with the N-terminal-specific (α -N; **A**, *3* and *6*; **B**, *2* and *4*) or C-terminal-specific (α -C; **A**, *4* and *7*; **B**, *3* and *5*) antisera or without added antisera (**A**, *2* and *5*). In both panels, MW standards are in *1* and the 185 kDa precursor is denoted on the right margin. Additionally, in **B** two potential products of 107 kDa and 78 kDa are denoted

anti-FLAG monoclonal antibody when expressed from pTM1/N-FLAG, but not when expressed from pTM1/C-FLAG, indicating that they are premature termination products. Enhancement of the detection of potential in vitro processing products in pTM1HEV programmed reactions was attempted using immunoprecipitation (Fig. 2B), pulse-chase radiolabeling with chases of as long as 5 h, deletion analysis and by the addition of protease inhibitors without success. The addition of cofactors such as microsomal membranes and divalent cations also failed to induce detectable processing.

### *In vivo expression*

In vivo expression of the HEV ORF1 was initially accomplished using vvHEV; similar results were obtained by transfecting with pTM1HEV and this construct was used for site-specific mutagenesis. As shown in Fig. 3A, following standard radiolabeling periods (< 5 h) done three hours post-infection, only the 185 kDa product was observed. This result was reproduced in four cell lines, Chang Liver,

HeLa, Vero and HepG2. However, when labeling times were extended to 24 h initiated at 18 h post-transfection, in addition to the 185 kDa product, a 107 kDa product immunoprecipitated by both the N- and C-terminal specific antisera and a 78 kDa product immunoprecipitated only by the N-terminal specific antiserum were also observed. Because of the extended radiolabeling periods, the gels contained a number of additional bands also present in the control vvWT lanes. These results were reproduced in Chang Liver, HeLa, Vero and HepG2 cells. Subsequent experiments showed that when radiolabeling was done at 18 h post-transfection these processing products were detectable, although very faint, following radiolabeling periods as short as 3–5 h (data not shown). However, to ensure that the processing would be detectable, subsequent experiments were done using the prolonged radiolabeling periods.

Immunoprecipitation with the N- and C-terminal specific antisera indicated that the 78 kDa product was N-terminal within the NSP and the 107 kDa product was C-terminal [because each serum recognized roughly half of the NSP (see Fig. 1B), the N-terminal specific antiserum would be expected to immunoprecipitate both products]. This was confirmed when the pTM1-HEV/N-FLAG and C-FLAG constructs were expressed. As shown in Fig. 4A, the anti-FLAG Mab immunoprecipiated the 78 kDa N-FLAG product and the 107 kDa C-FLAG product. Two deletion mutations and a site-specific mutation were also made using pTM1HEV, as diagrammed in Fig. 1. pTM1HEV$\Delta$Bam contained nt 28 to 2959 of the HEV ORF1 and the 108 kDa protein expressed from this construct contains the first 978 aa of the HEV NSP while pTM1HEV$\Delta$S/H lacked 2079 to 2439 of the HEV ORF1 and the 172 kDa protein expressed from this construct lacks aa 684 to 804 of the HEV NSP. The sizes of the proteins produced from these constructs was confirmed by in vitro expression to be as expected (data not shown). As shown in Fig. 4B, pTM1HEV$\Delta$BamHI (lanes 3–4), which deleted the C-terminus of the NSP, produced the deleted full-length 108 kDa product and also produced the 78 kDa processing product, confirming the previous finding that this product originates from the N-terminus of the NSP. This result also localizes a putative virus-specific protease domain to within the first 800 amino acids of the NSP. In contrast, pTM1HEV$\Delta$S/H (lanes 5–6) produced its deleted full-length 172 kDa product, but no apparent processing products, indicating that the deletion contains either the cleavage site or a putative protease. The site-directed mutation, pTM1HEV/CtoG, changed $C_{483}$ to G. As this was a member of the predicted catalytic dyad, this mutation should abrogate cleavage if the prediction was accurate. However, expression of the pTM1HEV/CtoG construct resulted in production of the full-length 185 kDa product and both the 107 and 78 kDa processing products (Fig. 4C), identical to the expression of pTM1HEV, indicating that this $Cys_{483}$ is not a catalytic residue.

### *Sequence alignment*

With the finding that $Cys_{483}$ was not a catalytic residue, sequence alignment of this region of the NSP was redone to include HEV strains sequenced after the original

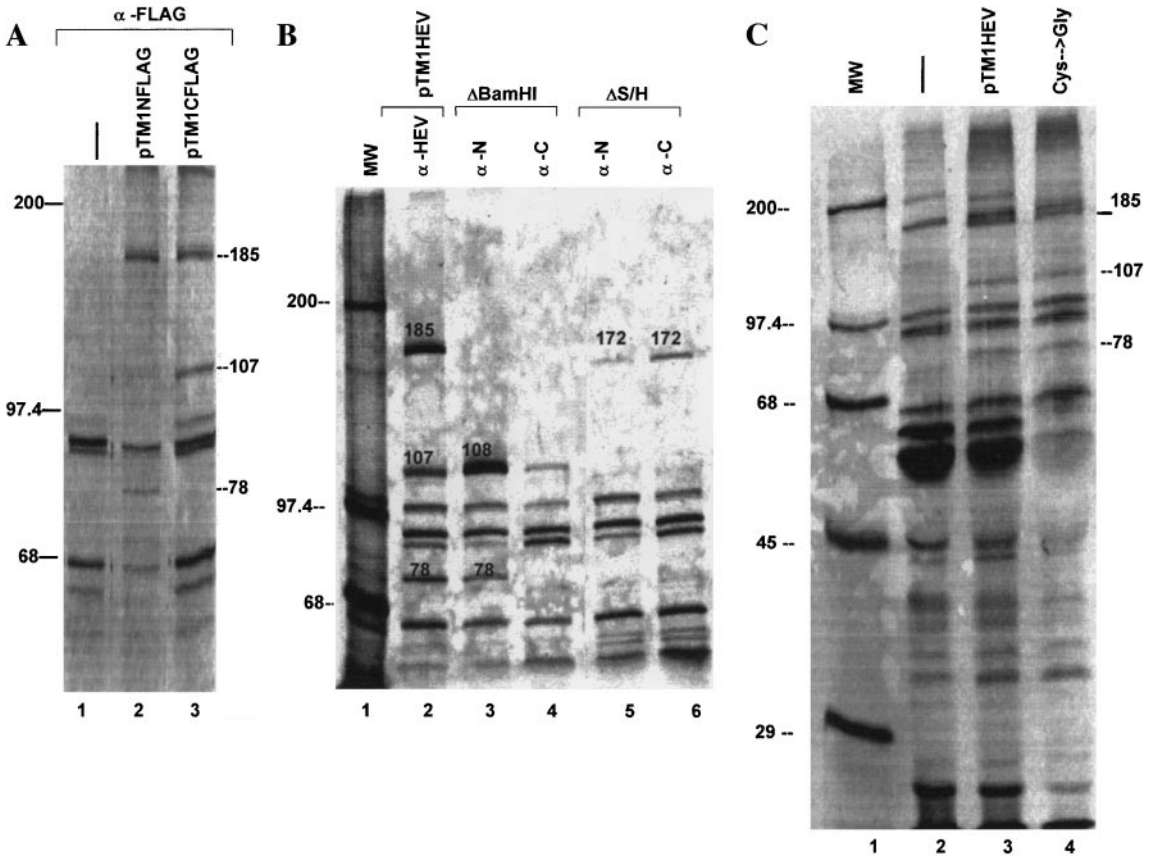



**Fig. 4.** In vivo expression by various pTM1HEV constructs. Monolayers of Chang liver cells were infected with vTF7-3 and transfected with the indicated constructs 12 h later. Radiolabeling was for 24 h at 18 h post-transfection. **A** Cells were mock-transfected (*1*) or transfected with pTM1HEV/N-FLAG (*2*) or pTM1HEV/C-FLAG (*3*). Lysates were immunoprecipitated with the anti-FLAG Mab. The 185 kDa, 107 kDa, and 78 kDa species are indicated on the right margin. **B** Cells were transfected with pTM1HEV (*2*), pTM1HEVΔBamHI (*3–4*), or pTM1HEVΔS/H (*5–6*). Lysates were immunoprecipitated with N-terminal-specific (α-N; *3* and *5*) or C-terminal-specific (α-C; *4* and *6*) antisera separately or combined (α-HEV; *2*). The sizes of the precursor produced by each construct (185 kDa for pTM1HEV, 108 kDa for pTM1HEVΔBamHI, and 172 kDa for pTM1HEVΔS/H) as well as the 78 kDa and 107 kDa cleavage products, if produced, are indicated over the relevant band. **C** Cells were mock transfected (*2*) or transfected with pTM1HEV (*3*) or pTM1HEV/CtoG (*4*). Lysates were immunoprecipitated with the N-terminal and C-terminal-specific antisera combined. The 185 kDa, 107 kDa, and 78 kDa species are indicated on the right margin. In **B** and **C**, MW standards are in *1* and in all three panels, the MWs are denoted on the left margin.

Three prominent products of 185, 107 and 78 kDa are indicated in the right margin

```
          472        ↓(483)     496   577          ↓(590)   601
MEXICO    CFMKWLGQECSCFLQPAEGLAGDQG...VDGARLEVNGPEQLNLSFDSQQCSM
CHINA     CFMKWLGQECTCFLQPAEGVVGDQG...VDGAVLETNGPERHNLSFDASQSTM
PAKISTAN CFMKWLGQECTCFLQPAEGVVGDQG...VDGAVLETNGPERHNLSFDASQSTM
BURMA     CFMKWLGQECTCFLQPAEGAVGDQG...VDGAVLETNGPERHNLSFDASQSTM
MYANOMAR CFMKWLGQECTCFLQPAEGVVGDQG...VDGAVLEANGPERYNLSFDASQSTM
```

**Fig. 5.** Alignment of the proposed PCP domain in 5 different HEV strains. An amino acid sequence alignment (aa residues 392 to 790 of ORF1) was made using HEV sequences available from GenBank (accession numbers M74506, Q03495, L25595, P33424, L08816, M73218, P29324, D10330 and Q04610) with the Pileup program from the GCG software package. The catalytic residues ($Cys_{483}$ and $His_{590}$) predicted by Koonin et al. [16] are indicated by arrows

alignment [16]. This alignment is shown in Fig. 5; the catalytic residues predicted by Koonin et al. [16] are indicated by an arrow. While $Cys_{483}$ is conserved among all of these HEV strains, $His_{590}$ is not. In the Mexico strain an L is found at residue 590 and in the Myanomar strain this residue is a Y. Thus, $His_{590}$ is also unlikely to be a catalytic residue.

### *Immunoprecipitation of lysates from HEV-infected hepatocytes*

In an attempt to confirm that the processing of the NSP observed when ORF1 was expressed also occurred in HEV-infected cells, immunoprecipitation using HEV NSP-specific antisera (HEV NSP-N- and C-terminal specific antisera combined) was done with lysates of radiolabeled HEV-infected and uninfected primary hepatocytes. However, no unique bands were detected in the HEV-infected cell lysates that were not also present in the uninfected cell lysates (data not shown). This result was not unexpected because Tam et al. [32] have been unable to detect HEV structural proteins in such lysates, and the presence of HEV can only be detected by the very sensitive method of RT-PCR.

## Discussion

The replication cycles of all positive-stranded RNA animal viruses studied include dependence on proteolytic processing by virally-encoded proteases. This study was designed to determine if HEV employed a similar strategy in the expression of its NSP. In addition to characterization of the HEV replication cycle, an HEV-specific protease would be an inviting target for anti-viral therapy during HEV infection.

In the absence of a routine cell culture system, the HEV ORF1, which encodes the NSP, was expressed in vitro using a coupled transcription/translation reticulocyte lysate system and in vivo using a vaccinia virus-driven transient expression system. Both systems have been used successfully in the characterization of a large number of plus-strand virus proteases of all classes (e.g. [1, 5, 9, 10, 15, 19, 22, 24, 30, 31, 34]). Two independent HEV Burmese strain constructs were employed in these studies. One construct, pBSORF1, which was used to gener-

ate pTM1HEV and vvHEV, was constructed with clones used in determination of the originally reported Burmese strain sequence [32]. The second construct, 625DNA, had six amino acid changes out of a total of 1693 aa in the NSP in comparison to the original Burmese strain sequence. Of these, T1077G (a T at aa 1077 in place of G in the originally reported sequence), C1088W, A1132P, A1254V, and R1315G were in the helicase region while L1543F was in the replicase region; none of these were near the predicted PCP. Interestingly, complete sequencing of the ORF1 sequence in pTM1HEV also revealed the presence of C1088W, A1132P, R1315G, and L1543F, indicating that these residues may be the authentic Burmese strain sequence. Additionally, A1077G was present in the pTM1HEV sequence.

Expression of ORF1 in vitro as well as in vivo under standard conditions yielded only the full-length 185 kDa NSP. Employment of conditions in vitro shown to activate other viral proteases, namely the presence of divalent cations [19, 31] and microsomoal membranes [34], were without effect. When in vivo expression was allowed to proceed for extended times, two ORF1-specific products of 78 and 107 kDa were detected. Since the size of these products added to the size of the NSP and these products came from the N- and C-termini of the NSP, respectively, their presence was consistent with proteolysis at a single site within the NSP. However, the PCP predicted in ORF1 was not involved in production of these products since mutagenesis of the predicted $Cys_{483}$ catalytic site had no effect on the cleavage. Considering that the other predicted catalytic residue, $His_{590}$, is not conserved across HEV strains, it seems doubtful that this PCP exists. Although predictions derived from computer alignments have often been accurate, there are other examples of when such predictions of proteases did not turn out to be correct [28]. Additionally, it is to be pointed out that in the prediction of the ORF1-PCP, it was noted that the alignment used to predict this PCP was weak [16]. The lack of a PCP within the HEV NSP is novel within the alphavirus-like superfamily in that the other animal virus members (RUB and the alphaviruses) utilize PCPs to catalyze cleavage of their NSP.

We were unable to find alignments between proteases of other classes and sequences within the HEV NSP using available databases and standard programs (FastA and Findpatterns of GCG; data not shown), similar to the findings of Koonin et al. [16]. Therefore, if one exists, an ORF1-specific protease would have to be of a novel type and its identification and localization would require extensive site-directed mutagenesis (the deletion analysis done in this study localized the putative protease to the amino-terminal half of the NSP). It is also possible that the cleavage of the NSP observed following prolonged in vivo expression was mediated by an enzyme expressed by the vaccinia virus vector or by a ubiquitous cellular enzyme (cleavage was observed in four different cell lines). The former has not previously been encountered during vaccinia virus expression of other viral NSPs which are processed by their own enzymes, but could be checked by expressing ORF1 with a different system. The latter is consistent with the lack of processing in vitro, but has not been described in the processing of other

viral NSPs; it could also be an artifact of prolonged expression. Dependence of NSP cleavage on a cellular enzyme would effect both the host range and tissue tropism of HEV. However, we were unable to authenticate this cleavage in HEV-infected primary hepatocytes. Whatever the source of the enzyme, the NSP cleavage reaction observed during prolonged in vivo expression was exceedingly slow and/or inefficient.

The order of the cleavage products within the NSP is N-78kDa-107kDa-C which would place the cleavage site at roughly aa 700. Consistent with this placement, the pTM1HEV$\Delta$S/H deletion mutant which lacked residues 684 to 804 failed to exhibit cleavage. If this cleavage is authentic, the HEV MT and Y domains would be localized on the 78 kDa product and the proline hinge, X, helicase, and replicase domains on the 107 kDa product. The presence of the helicase and replicase domains on the same protein would be similar to RUB with which HEV is phylogenetically most closely related and unique to these two viruses among animal viruses of the alpha-like superfamily.

In summary, we were unable to observe cleavage of the HEV NSP using both standard in vitro and in vivo conditions under which most virally-encoded proteases function. However, cleavage was observed following prolonged in vivo expression but it could not be authenticated because we were unable to detect ORF1-specific products in HEV-infected primary monkey hepatocytes, one of only two reported cell culture systems for HEV. Therefore, the HEV NSP may not be cleaved at all, but if the cleavage following prolonged expression is authentic, it is putatively mediated by a novel virally-encoded protease or a cellular protease. An efficient culture system for HEV will be the most direct means for resolving these questions and when such becomes available, the reagents and constructs produced during this study should be very useful in this regard.

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## References

1. Alonso JMM, Casais R, Boga JA, Parra F (1996) Processing of rabbit hemorrhagic disease virus polyprotein. J Virol 70: 1261–126
2. Bradley DW (1995) Hepatitis E virus: a brief review of the biology, molecular virology and immunology of a novel virus. J Hepatol 22: 140–145
3. Bradley DW, Krawczynski K, Cook Jr EH, McCaustland KA, Robertson BJ, Humphrey CD, Kane MA, Spelbring J, Weisfuse I, Andjaparidze A, Balayan M, Stetler H, Velazquez O (1988) Enterically transmitted non-A, non-B hepatitis: etiology of disease and laboratory studies in nonhuman primates. In: Zuckermann AJ (ed) Viral hepatitis and liver disease. A.R. Liss, New York, pp 1 238–1 247
4. Bradley DW, Beach MJ, Purdy MA (1993) Molecular characterization of hepatitis C and E viruses. Arch Virol 7: 1–14
5. Chambers JF, Grakoui A, Rice CM (1991) Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. J Virol 65: 6 042–6 050

6. Cubitt D et al (1995) Caliciviridae. In: Murphy FA, Fauquet CR, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MO (eds) Virus Taxonomy. Classification and Nomenclature of Viruses. Sixth Report of the International Committee on Taxonomy of Viruses. Springer, Wien New York, pp 359–363 (Arch Virol [Suppl] 10)
7. Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12: 387–395
8. Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res 16: 6 127–6 145
9. Falgout B, Pethel M, Zhang Y-M, Lai C-J (1991) Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J Virol 65: 2 467–2 475
10. Hardy WR, Strauss JH (1989) Processing the nonstructural polyproteins of Sindbis virus: Nonstructural proteinase is in the C-terminal half of nsP2 and functions both in cis and in trans. J Virol 63: 4 653–4 664
11. Hehn A, Fritsch C, Richards KE, Guilley H, Jonard G (1997) Evidence for in vitro and in vivo autocatalytic processing of the primary translation product of beet necrotic yellow vein virus RNA1 by a papain-like proteinase. Arch Virol 142: 1 051–1 058
12. Huang R, Nakazono N, Ishii K, Li D, Kawamata O, Kawaguchi R, Tsukada Y (1995) Hepatitis E virus (87A Strain) propagated in A549 cells. J Med Virol 47: 229–302
13. Kane MA, Bradley DW, Shrestha SM, Maynard JE, Cook EH, Mishra RP, Joshi DD (1984) Epidemic non-A, non-B hepatitis in Nepal: recovery of a possible etiologic agent and transmission studies in marmosets. JAMA 252: 3 140–3 145
14. Khuroo MS (1980) Study of an epidemic of non-A, non-B hepatitis: possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. Am J Med 68: 818–824
15. Kirchweger R, Ziegler E, Lamphear BJ, Water D, Liebig HD, Sommergruber W, Sobrino F, Hohenadl C, Blaas D, Rhoads RE (1994) Foot-and-mouth disease virus leader proteinase: purification of the Lb form and determination of its cleavage site on eIF-4 gamma. J Virol 68: 5 677–5 684
16. Koonin EV, Gorbalenya AE, Purdy MA, Rozanov MN, Reyes GR, Bradley DW (1992) Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. Proc Natl Acad Sci USA 89: 8 259–8 263
17. Krausslich H-G, Wimmer E (1988) Viral proteinases. Ann Rev Biochem 57: 701–754
18. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
19. Liu X, Ropp SL, Jackson RJ, Frey TK (1998) The rubella virus nonstructural protease requires divalent cations for activity and functions in trans. J Virol 72: 4 463–4 466
20. Mackett M, Smith GL, Moss B (1984) General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J Virol 49: 857–864
21. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
22. Marr LD, Wang C-Y, Frey TK (1994) Expression of the rubella virus nonstructural protein ORF and demonstration of proteolytic processing. Virology 198: 586–592
23. Moss B, Elroy-Stein O, Mizukami T, Alexander WA, Fuerst TR (1990) New mammalian expression vectors. Nature 348: 91–92
24. Preugschat F, Strauss JH (1991) Processing of nonstructural proteins NS4A and NS4B of Dengue 2 virus in vitro and in vivo. Virology 185: 680–697
25. Pringle CR (1998) Virus taxonomy: San Diego 1998. Arch Virol 143: 1 449–1 459

26. Purdy MA, Tam AW, Huang C-C, Yarbough PO, Reyes GR (1993) Hepatitis E virus: a non-enveloped member of the 'Alpha-like' RNA virus supergroup? Semin Virol 4: 319–326
27. Reyes GR, Purdy MA, Kim JP, Luk K-C, Young LM, Fry KE, Bradley DW (1990) Isolation of a cDNA from the virus responsible for enterically transmitted non-A non-B hepatitis. Science 247: 1 335–1 339
28. Rumenapf T, Stark R, Heimann M, Thiel H-J (1998) N-terminal protease of pestiviruses: Identification of putative catalytic residues by site-directed mutagenesis. J Virol 72: 2 544–2 547
29. Shrestha SM, Malla DS (1975) Viral A hepatitis in pregnancy during Kathmandu epidemic. J Nepal Med Assoc 13: 58–69
30. Snijder EJ, Wassenaaur ALM, Spaan WJM (1994) Proteolytic processing of the replicase ORF1A protein of equine arteritis virus. J Virol 68: 5 755–5 764
31. Stempniak M, Hostomska Z, Nodes B, Hostomsky Z (1997) The NS3 proteinase domain of hapatitis C virus is a zinc-containing enzyme. J Virol 71: 2 881–2 886
32. Tam AW, Smith MM, Guerra ME, Huang C-C, Bradley DW, Fry KE, Reyes GR (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length genome. Virology 185: 120–131
33. Tam A W, White R, Yarbough PO, Murphy BJ, McAtee CP, Lanford RE, Fuerst TR (1997) In vitro infection and replication of hepatitis E virus in primary cynomolgus macaque hepatocytes. Virology 238: 94–102
34. Tibbles KW, Brierley I, Cavanagh D, Brown TDK (1996) Characterization in vitro of an autocatalytic processing activity associated with the predicted 3C-like proteinase domain of the coronavirus avian infectious bronchitis virus. J Virol 70: 1 923–1 930
35. Uchida T, Suzuki K, Komatsu K, Iida F, Shikata T, Rikihisa T, Mizuno K, Soe S, Win KM, Nakane K (1990) Occurrence and character of a putative causative virus of enterically-transmiited non-A non-B hepatitis in bile. Jpn J Exp Med 60: 23–29
36. Wellink J, vanKammen A (1988) Proteases involved in the processing of viral polyproteins: brief review. Arch Virol 98:1–26
37. Zafrullah M, Ozdener MH, Panda SK, Jameel S (1997) The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J Virol 71: 9 045–9 053

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