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The Open Reading Frame 3 Gene of Hepatitis E Virus Contains a *cis*-Reactive Element and Encodes a Protein Required for Infection of Macaques

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An infectious cDNA clone of hepatitis E virus was mutated in order to prevent synthesis of either open reading frame 2 (ORF2) protein or ORF3 protein. HuH-7 cells transfected with an ORF2-null mutant produced ORF3, and those transfected with an ORF3-null mutant produced ORF2. Silent mutations introduced into a highly conserved nucleotide sequence in the ORF3 coding region eliminated the synthesis of both ORF2 and ORF3 proteins, suggesting that it comprised a *cis*-reactive element. A mutant that was not able to produce ORF3 protein did not produce a detectable infection in rhesus macaques. However, a mutant that encoded an ORF3 protein lacking a phosphorylation site reported to be critical for function was able to replicate its genome in cell culture and to induce viremia and seroconversion in rhesus monkeys, suggesting that phosphorylation of ORF3 protein was not necessary for genome replication or for production of infectious virions.

Hepatitis E virus (HEV) is a major cause of acute viral hepatitis in many developing countries, where it is responsible for massive waterborne epidemics, as well as sporadic cases of hepatitis E (10). Although originally thought to be virtually absent from most industrialized countries, sporadic cases are becoming more commonly recognized, probably due to increased awareness and better diagnostic tests. The relatively high seroprevalence of anti-HEV in normal populations in the industrialized countries coupled with a very high seroprevalence in numerous animal species, including rodents, swine, and chickens, has lent credence to the hypothesis put forward by Balayan many years ago that hepatitis E is a zoonotic disease (7). Recently, convincing evidence that HEV might be transmitted by eating raw or undercooked meat was obtained (15, 21).

HEV is a nonenveloped virus with a single-stranded RNA genome of 7.2 kb (10). The genome is positive sense and contains three open reading frames (ORFs). The 5′ two-thirds of the genome contains ORF1, which encodes nonstructural proteins involved in viral RNA synthesis. ORF2, the capsid gene, occupies the 3′-terminal part of the genome and ORF3, which overlaps both ORF1 and ORF2, encodes a very small protein containing only 123 amino acids.

HEV has been very difficult to grow in cell culture, so only the most basic knowledge of its molecular biology has been obtained. The genome is capped and polyadenylated, and the first step of infection after uncoating and release of the viral genome into the cytoplasm is expected to be translation of ORF1. ORF2 and ORF3 proteins are believed to be encoded by separate but 3′-coterminal subgenomic RNAs (14, 20), which would need to be synthesized by the newly translated viral RNA-dependent RNA polymerase (RdRp): their termini have not yet been mapped, and it is not known how their synthesis or translation is regulated.

ORF2 protein is the major, if not only, viral capsid protein and as such would be required for virion production and infectivity. In vitro vector-based expression studies suggest recombinant ORF2 protein is posttranslationally modified by proteolytic cleavage at one or more sites (11, 24) and by glycosylation at one to three sites (4, 16, 22). Recombinant ORF2 protein is found both intracellularly and on the cell surface (4, 22). The size and glycosylation state of ORF2 protein assembled into infectious virions is unknown. Yeast three-hybrid assays have shown that ORF2 protein can bind to the 5′ region of HEV genomes, an interaction that might be involved in genome encapsidation during virion formation (13).

Even less is known about ORF3 protein. It is not known even if ORF3 protein is a component of virions. All information about ORF3 function to date derives from plasmid-based, high-level expression of recombinant protein. Recombinant ORF3 protein is a phosphoprotein that segregates with the cytoskeleton in cell fractionation studies (23). Recombinant ORF3 and ORF2 proteins interacted in fluorescence-based colocalization studies, yeast two-hybrid experiments, and coimmunoprecipitation assays (17): this interaction required phosphorylation of Ser 80, the only amino acid of ORF3 protein known to be phosphorylated (17). ORF3 protein interacted preferentially with full-length, nonglycosylated ORF2 protein, leading to the speculation that ORF3 protein may have a role in virion assembly.

ORF3 is believed to interact also with nonviral proteins. Binding assays have demonstrated that recombinant ORF3

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protein binds via a proline-rich region to the src homology 3 domain of cellular proteins involved in signal transduction (5, 6): hence, ORF3 protein has been postulated to be a viral regulatory protein that modulates cell signaling. Because a reliable and robust cell culture system is not available, none of these functions has yet been confirmed under more natural conditions reflecting viral replication and particle assembly.

The development of a cDNA clone that can be transcribed in vitro to produce HEV RNA genomes that are infectious for cultured cells and for nonhuman primates provided a means to study the molecular biology of HEV under more normal conditions (1, 2). We have mutated here this infectious clone to try to determine whether ORF3 protein has a critical role in HEV replication or infection. In the course of these studies, we have identified a second *cis*-reactive element in the coding region of the genome.

MATERIALS AND METHODS

Cells. HuH-7 cells, a human hepatoma cell line (8) were grown at 37°C in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum (ultra-low immunoglobulin G [IgG]; Invitrogen) that was heat inactivated at 56°C for 30 min (DMEM [5%]).

Mutant construction. The infectious cDNA clone of the Sar 55 strain, pSK-HEV-2 (GenBank accession no. AF 444002) was used as the parent clone for all mutants. The replicon pE/luc (ORF2/luciferase) has been described (3) and was constructed by replacing nucleotides (nt) 5148 to 5816 of pSK-HEV-2 with the luciferase gene. Inactivation of an RdRp by introduction of a GAD mutation into the GDD catalytic site has been described (1, 13, 19). Mutations were introduced by standard methods of oligonucleotide site-directed mutagenesis, fusion PCR, and substitution of mutated fragments into the full-length clone or replicon. The entire HEV sequence was determined after mutagenesis to verify that unwanted mutations had not been introduced.

In vitro transcription and transfection. Plasmids were linearized at a unique BglII site located immediately downstream of the poly(A) tract of the HEV sequence. Capped transcripts were synthesized with the T7 riboprobe in vitro transcription system (Promega) in the presence of cap analog as previously described (1). Each 50-μl reaction contained 10 μl of 5× transcription buffer, 5 μl of 100 mM dithiothreitol, 2 μl of 40 U of RNasin/ml, 5 μl of nucleoside triphosphates (5 mM [each] ATP, CTP, and UTP and 0.5 mM GTP), 5 μl of 5 mM $m^7G(5')$ ppp(5′)G (Ambion), and 2 μl of 20 U/μl T7 polymerase. The mixtures were incubated at 37°C for 1.5 to 2 h, with an additional 2 μl of polymerase added after the first hour. The integrity and yield of transcripts were determined by electrophoresis on a nondenaturing agarose gel. Transcription mixtures were cooled on ice and then mixed with a liposome mixture (20 μl of DMRIE-C [Invitrogen]/ml of OptiMem [Gibco]) for transfection of HuH-7 cells. A total of 10 μl of RNA mixture was diluted with 200 μl of the liposome mixture and added to one well of a 12-well plate or a 2-well chamber slide. Volumes were doubled for a six-well plate or reduced to 7.5 μl of RNA mixture/150 μl of liposome mixture for a 24-well plate. In all cases, the cells were incubated at 34.5°C for 5 h; the transfection mixture was then replaced with DMEM (5%), and the incubation was continued at 34.5°C. Cells that were not in chamber slides were treated with trypsin and replated in chamber slides 1 to 2 days prior to immunostaining.

Luciferase assay. Transfected cells were washed twice with phosphate-buffered saline (PBS) and harvested into 200 μl of 1× passive lysis buffer (Promega). The lysates were immediately frozen at −80°C. The samples were freeze-thawed twice and clarified by centrifugation for 2 min; a 20-μl aliquot of each cell extract was then tested for luciferase activity with the luciferase assay system (Promega) according to the manufacturer's instructions. A TRILUX 1450 luminescence counter (Perkin-Elmer) was used for detection.

Transfection of rhesus macaques. Capped RNA was transcribed in a 200-μl volume with the Promega T7 riboprobe in vitro transcription system as described above or, alternatively, with the T7 Megascript kit (Ambion) according to the manufacturer's instructions. A total of 5 μl was removed for gel electrophoresis, and the remaining 195 μl of transcription mixture was diluted with 805 μl of PBS without calcium and magnesium and immediately frozen on dry ice. Within 24 h. the mixture was thawed and injected into multiple sites in the liver of a rhesus macaque by percutaneous intrahepatic injection guided by ultrasound as described previously (2). Macaques were prescreened for antibodies to HEV with a very sensitive enzyme-linked immunosorbent assay (ELISA) (2) in order to ensure that they would be susceptible to HEV infection. Each mutant was tested in two macaques simultaneously, and the experiment was repeated if the animals did not get infected. The intrahepatic protocol has proved to be very reliable and reproducible. The normal course of infection occurred in four of four rhesus monkeys after inoculation of the wild-type clone (2; J. Graff, unpublished data) and in each of two monkeys after inoculation with three different mutants ((3) and the present study) for a total of 10 successful infections out of 10 attempts. Sera were monitored weekly for serum alanine amino transferase (ALT) levels (Anilytics, Gaithersburg, MD), anti-HEV, and viral genomes. ALT levels ≥2 times the mean preinoculation levels were considered evidence of hepatitis. The preinoculation level was calculated as the geometric mean of three weekly samples collected at week 0 and before inoculation. HEV antibodies were detected with an in-house ELISA as described previously (2). Viral genomes were quantified by HEV-specific real-time reverse transcription-PCR (RT-PCR) from total RNA extracted from 100 μl of serum as previously described (2).

The animals were housed at Bioqual (Rockville, MD). The housing, maintenance, and care of the animals met or exceeded all requirements for primate husbandry as specified in the *Guide for the Care and Use of Laboratory Animals* (9).

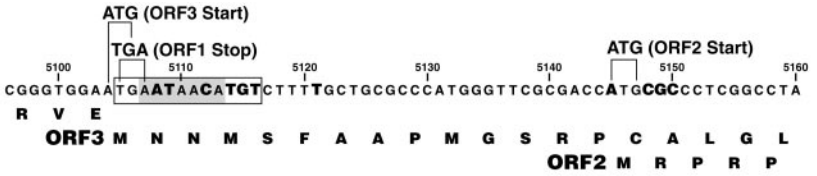
Sequence analysis. Genome fragments amplified by RT-PCR from serum of inoculated animals were purified after agarose gel electrophoresis (QiaQuick; Qiagen) and sequenced directly with an automated sequencer. RNA structural predictions were performed with the MFOLD program of the GCG suite (Wisconsin Package, version 10.0; Genetics Computer Group) (25).

Immunofluorescence microscopy. Cells were fixed with acetone and air dried as described previously (1). After fixation, the cells were incubated for 20 min at room temperature with a 1:1 mixture of 10% bovine serum albumin and PBS containing a mixture of mouse monoclonal (MAb) or rabbit polyclonal anti-ORF3 and chimpanzee monoclonal or polyclonal anti-ORF2. Mouse α-ORF3 MAb, described previously (1) was a gift from Isa Mushahwar at Abbott Laboratories. This antibody reacts in ELISA with a synthetic peptide containing amino acids 91 to 123 of ORF3 of the Sar 55 strain. Anti-ORF3 polyclonal antibody was produced in rabbits immunized with a synthetic peptide comprising amino acids 91 to 123 of ORF3 of the human HEV strain Sar 55 (Lofstrand). The anti-ORF2 MAb was derived from a chimpanzee by panning a phage-display library with recombinant ORF2 protein (12). The polyclonal anti-ORF2 was derived from a chimpanzee (animal 1313) that had been sequentially inoculated with the Sar 55 strain and the Mex-14 strain of human HEV, respectively: the ELISA titer against recombinant ORF2 protein was below the limit of detection (<1:20) for the preinoculation serum and was $1:10^5$ postinoculation. The polyclonal serum was specific for ORF2 protein and precipitated ^{35}S-labeled ORF2 protein but not ^{35}S-labeled ORF3 protein translated in vitro. There was virtually complete colocalization of ORF2 protein stained with the monoclonal and the polyclonal antibodies.

After a wash in PBS, the slides were incubated for 20 min at room temperature with a mixture of Alexa Fluor 488-conjugated goat anti-human IgG (Molecular Probes) and Alexa Fluor 568-conjugated goat anti-rabbit or goat anti-mouse IgG (Molecular Probes) as the secondary antibodies. The samples were washed in PBS, Vectashield (Vector Laboratories) was added, and the slides were viewed by indirect immunofluorescence microscopy with a fluorescein isothiocyanate filter set for Alexa Fluor 488 (green), a rhodamine filter set for Alexa Fluor 568 (red), and the 25× objective of a Zeiss fluorescence photomicroscope. Alternatively, the samples were subjected to confocal microscopy at the Biological Image Facility at the National Institute of Allergy and Infectious Diseases.

RESULTS

Nonhuman primates transfected intrahepatically with capped transcripts from a full-length cDNA clone of HEV exhibit a normal course of infection, including viremia, hepatitis, and seroconversion to the HEV capsid protein, thus demonstrating that the recombinant genome is functional and replicating normally (2). Unfortunately, although transfection of cultured human liver cells with capped transcripts derived from this same clone resulted in production of virus that was infectious for rhesus macaques, the virus did not spread within the cell cultures (1), probably because the appropriate cell receptor was missing. This lack of spreading, coupled with the fact



Nucleotide Mutation	Amino Acid Changes	Protein Detected	
		ORF2	ORF3
A5145, 5178, 5190→C G5676→T (AA) T5690→(TA) G	ORF2 M→L, possible tripeptide (aa175-177) ORF3 unchanged	No	Yes
Delete A5108	ORF2 unchanged ORF3 frame shift	No	No
T5109→C C5112→U TCT 5116-5118→AGC T5121→C	ORF2 unchanged ORF3 unchanged	No	No
CGC 5148-5150→AGA	ORF2 unchanged ORF3 termination codon (TGA) at AA 15	Yes	No

FIG. 1. Schematic diagram of part of ORF3 overlap regions indicating introduced mutations and location of a conserved nucleotide sequence. The nucleotide and amino acid sequences for pSK-HEV-2 are shown (GenBank no. AF444002). Box: region conserved among human HEV strains (GenBank nos. AY115488, AP003430, AB074918, AB073912, AF082843, AF060668, AF060669, AB074920, AB097812, D10330, M94177, AF076239, E17109, X99441, AF459438, M73218, D11092, L25547, X98292, AJ272108, AF444003, AY230202, M74506, AB091395, AB091394, AB080575, AB074915, AB074917, and AB097811). The shaded box indicates seven contiguous nucleotides identical in human and avian HEV(GenBank no. AY043166).

that this virus does not have a robust replication cycle and that successful transfection thus far has been limited to only 10% of the cells in the culture, has prevented us from detecting newly synthesized viral RNAs. Attempts to perform real-time RT-PCR or Northern blotting all have been confounded by the presence of excess input RNA: since the capping reaction is inefficient and a cap is required for infectivity (1, 2), large amounts of transcripts need to be transfected. However, we have been able to detect ORF2 or ORF3 proteins or reporter proteins expressed in their stead (1, 3). Since these proteins are not detected if the RdRp is selectively inactivated by the introduction of a single amino acid mutation into its catalytic site (1, 3), the production of these proteins is dependent on viral RNA synthesis and can be used as a surrogate assay for viral RNA replication.

Synthesis of ORF3 protein. A replicon in which ORF3 was truncated to contain only the first 42 nt and ORF2 was replaced with the gene for green fluorescent protein (GFP) produced GFP in transfected cells but only when the replicon encoded the wild-type RdRp: GFP was not detected if the catalytic site of the polymerase gene was mutated (1). This result suggested that GFP production required viral RNA replication and that neither ORF2 nor ORF3 protein was required for viral RNA synthesis in vitro. However, recombinant ORF2 and ORF3 proteins have been shown to bind to each other in molecular assays (17), so it was conceivable that genomic expression of one in the absence of the other might result in a novel phenotype.

The partial overlap of reading frames 1 and 3 and of reading frames 3 and 2 restricted the type and placement of mutations that could be introduced. Therefore, in order to abolish ORF2 protein synthesis without affecting that of ORF3, the first three methionine codons (encoding amino acids 1, 12, and 16) in ORF2 of the full-length infectious cDNA clone were mutated to encode leucine (ATG→CTG) and two in-frame stop codons were introduced just downstream of the fourth methionine codon at amino acid 175 (Fig. 1). None of the mutations changed the amino acid sequence of the ORF3 protein. Capped in vitro transcripts from this mutant, ΔORF2, and from the wild-type infectious clone were transfected in parallel into HuH-7 cells.

Transfected cells were examined by confocal immune fluorescence microscopy after being stained with a mixture of antisera specific for ORF2 and ORF3 proteins, respectively. By day 7 posttransfection, cell cultures transfected with the wild-type genomes contained many stained cells and both ORF2 and ORF3 proteins were clearly detected in the majority of them (Fig. 2A and B). In contrast, parallel cultures transfected with the ΔORF2 genomes contained a similar number of stained cells by day 7 posttransfection, but only ORF3 protein was detected (Fig. 2D and E): cells transfected with ΔORF2 were not stained with either an MAb to ORF2 protein or with

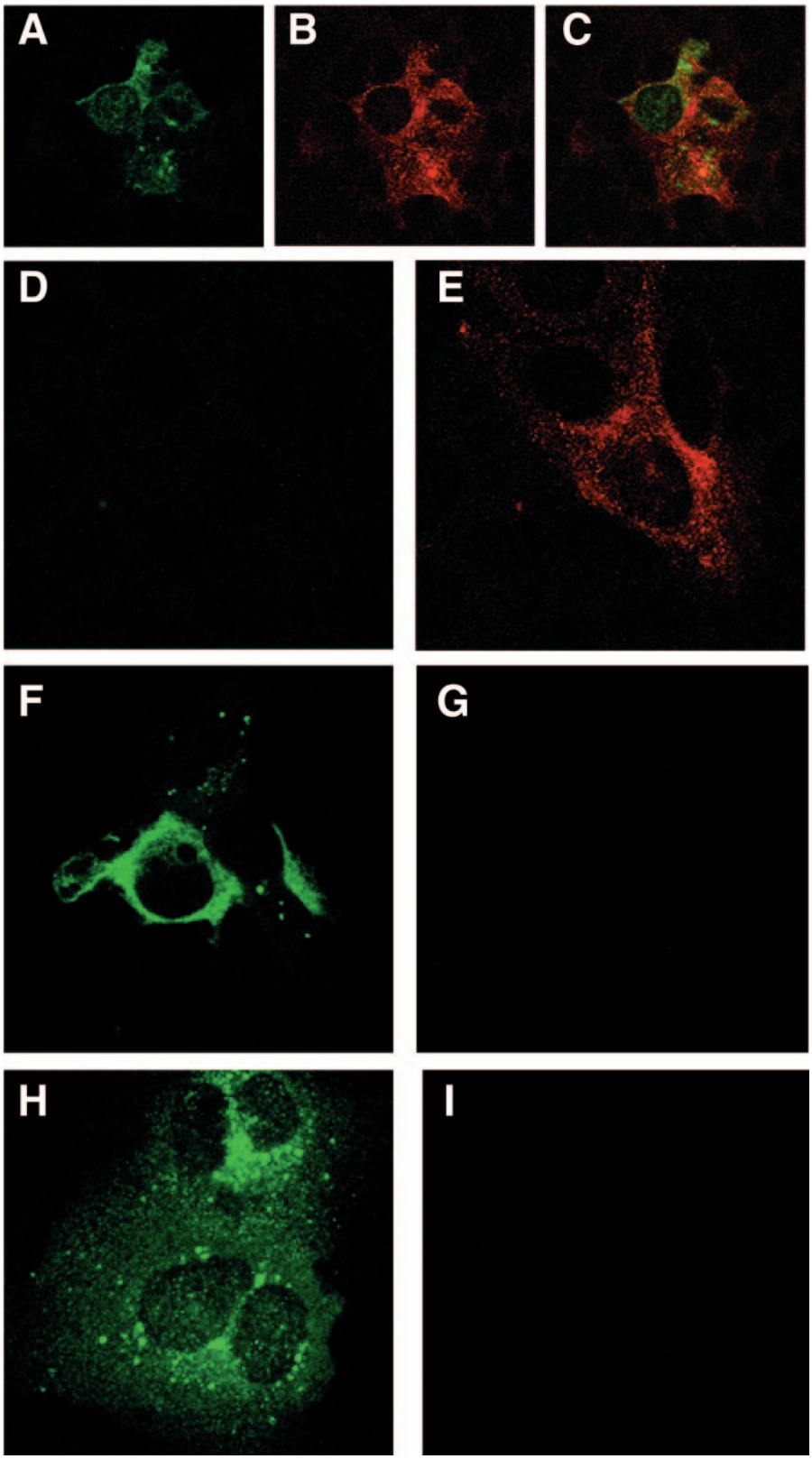


FIG. 2. Confocal immune fluorescence microscopy demonstrating that ORF2 protein (green) is not detected in HuH-7 cells transfected with ΔORF2 genomes and ORF3 (red) is not produced in cells transfected with an ORF3-null mutant. Note that the generation of ORF2 granules is a slow and asynchronous process so that cells within one transfected sample exhibited the total range of staining patterns and the cells shown portray a single example in each case. Cells transfected with wild-type genomes and stained on day 7 posttransfection with MAb α-ORF2 (A) or MAb α-ORF3 (B) (merge image [C]). Cells transfected with ΔORF2 genomes and stained on day 7 posttransfection with MAb α-ORF2 (D) or MAb α-ORF3 (E). Cells transfected with an ORF3-null mutant and stained on day 7 posttransfection with α-ORF2 MAb (F), α-ORF3 MAb (G), α-ORF2 polyclonal antibody (H), or α-ORF3 MAb (I). All photographs were taken with the 63× objective lens, but panels A to C were reduced compared to panels D to I for graphic layout.

TABLE 1. Anti-HEV in rhesus macaques after transfection[a]

Rhesus ID (virus genomes)[b]	Peak OD/cutoff ratio[c]
H624 (wild type)	**436.0**
H622 (wild type)	**204.0**
CK57 (ORF3 frameshift)	0.4
CK58 (ORF3 frameshift)	0.4
CJ7A (ORF3 null)	0.5, 0.5*
CJ7R (ORF3 null)	0.4, 0.5*
H666 (S80A)	0.3, **3.4***
H667 (S80A)	0.4, 0.4*
H695 (S80L)	**149.0**
H696 (S80L)	**218.7**

[a] Antibody was detected by ELISA based on recombinant ORF2 protein.
[b] In vitro-transcribed genomes transfected. ID, identification.
[c] Peak ELISA optical density (OD) of serum times the dilution factor divided by the cutoff value: a ratio of ≥1.0 denotes seroconversion (boldface). *, Value for initial transfection, followed by value for repeat transfection.

a polyclonal immune serum specific for ORF2 protein. There was not an obvious difference in the cytoplasmic distribution of ORF3 protein in the presence or absence of ORF2 protein, which may reflect the fact that there was little, if any, colocalization of these two proteins observed when cells were transfected with the wild-type genome (Fig. 2C).

The staining confirmed that ORF2 protein was not synthesized and indicated that genomic expression of ORF3 protein did occur and that ORF3 protein accumulated in cells in the absence of ORF2 protein.

A frameshift mutation at the 5′ terminus of ORF3 abolished ORF2 protein production. In an attempt to inhibit ORF3 protein synthesis while permitting ORF2 protein synthesis, an ORF3/Fs mutant was generated, in which the fifth nucleotide (nt 5108) in ORF3 was deleted to shift the reading frame: this deletion was downstream of the ORF1 termination codon and upstream of the initiation codon for ORF2 so neither of these ORFs was altered (Fig. 1). Again, capped in vitro transcripts, this time from the mutant (ORF3/Fs) and the wild-type clone, were transfected in parallel into HuH-7 cells. Immune fluorescence microscopy of doubly stained cells was performed as before.

In agreement with the previous results, 5 to 10% of the cells in cultures transfected with the wild-type genomes stained with both anti-ORF2 and anti-ORF3 by day 6 posttransfection (data not shown). Unexpectedly, the cultures transfected with the ORF3 frame shift mutant did not contain cells that were stained, with either anti-ORF3 or anti-ORF2, at day 6 or 13 posttransfection. Since the ORF3/Fs mutant was identical to the wild type except for missing the single A residue and since the in vitro transcripts appeared to be quantitatively and qualitatively similar to those synthesized from the wild-type template at the same time and with the same reagents (data not shown), it appeared that the frameshift was lethal.

The nonviability of the mutant was confirmed by inoculating capped RNA transcripts intrahepatically into two rhesus macaques according to our standard procedure, which routinely results in infection with the wild-type clone. All sera collected weekly from both macaques (CK57 and CK58) through week 17 posttransfection remained negative for anti-ORF2 antibody (Table 1) (or ALT elevation), suggesting that this mutant was unable to replicate and/or spread in vivo.

The inability to detect evidence of viral replication either in vivo or in vitro could be explained in two ways. First, the frameshifted ORF3 had the potential to encode a nonsense protein of 55 amino acids (data not shown), and it was conceivable that this protein might somehow interfere with ORF2 protein synthesis or stability. Alternatively, the RNA sequence in this region might contain regulatory sequences and thus might not be able to tolerate mutations.

Sequence comparisons revealed that a continuous 12-nt region encompassing the A residue of interest was universally and totally conserved among the four HEV genotypes in all 28 of the full-length mammalian genome sequences available at this time (Fig. 1). Also, even though the avian HEV shares only ~50% nucleotide identity overall with the mammalian strains, seven contiguous residues in this region, including the mutated A residue, were identical to those of the mammalian strains.

Nucleotide sequence at the 5′ end of ORF3 affects ORF2 protein production. The strict nucleotide sequence conservation at the start of ORF3 suggested that the frameshift introduced into this region was lethal, not because it eliminated ORF3 protein production but because it impacted an RNA function. To test this hypothesis, we mutated 6 nt in and around this region (Fig. 1). These mutations were all outside of ORF1 and ORF2 coding regions and were predicted to alter RNA folding (Fig. 3) but not ORF3 amino acid sequence. In spite of the total conservation of the amino acid sequence of every viral protein, this mutant did not synthesize detectable ORF2 or ORF3 protein after transfection of HuH-7 cells. A variant of this mutant that contained only the last four (nt 5116, 5117, 5118, and 5121) of the six mutations also was unable to synthesize ORF2 or ORF3 protein in transfected cells (data not shown).

The inability of these two mutants to produce detectable ORF2 or ORF3 protein strongly suggested that the nucleotides in this region contributed to or constituted an essential *cis*-reactive element (CRE). However, the experiments did not solve the original problem of abolishing ORF3 protein synthesis while maintaining that of the ORF2 protein.

ORF2 protein synthesis in the absence of ORF3 protein. Since functional HEV replicons have been constructed by substituting either a GFP or luciferase reporter gene for the downstream sequence adjacent to the ORF2 translation initiation codon, it was clear that the nucleotide sequence in this region could be altered without preventing genomic replication (1, 3). Therefore, the second codon in ORF2 (CGC; nt 5148 to 5150) was mutated to AGA to keep arginine as the second amino acid in ORF2 protein while introducing a TGA termination codon into the ORF3 reading frame and leaving the upstream conserved sequence intact (Fig. 1). This mutant should produce authentic ORF2 protein and a truncated ORF3 protein of only 15 amino acids. When capped transcripts from this clone were transfected into HuH-7 cells, the number of cells stained with antibodies at day 7 posttransfection was comparable to that of the wild-type control but, whereas both ORF2 and ORF3 proteins were present in cells transfected with wild-type genomes (2A and 2B), only ORF2 was detected in cells transfected with the ORF3-null mutant genome (Fig. 2F to 2I).

The absence of ORF3 protein and the presence of ORF2 protein was confirmed in a repeat experiment in which the cytoplasmic distribution of ORF2 protein was assessed. We have found that for the first 4 to 6 days after ORF2 protein is

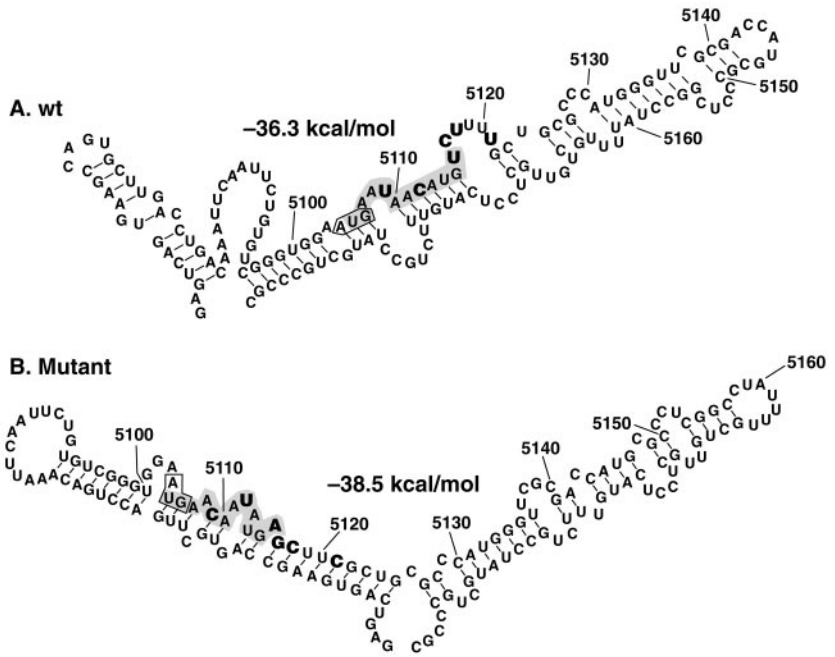


FIG. 3. Synonomous mutations that prevent synthesis of ORF2 and ORF3 proteins change the predicted secondary structure of the RNA. The first methionine codon in ORF3 is boxed. Six introduced synonymous mutations are indicated in boldface, and the conserved region is shaded. The predicted secondary structures with the lowest energy are presented.

detected in transfected cells, it is mostly distributed fairly uniformly in the cytoplasm, whereas after 7 days posttransfection, large granules of ORF2 antigen begin to accumulate in an asynchronous manner (see Fig. 2H for beginning granule formation). This changing cytoplasmic distribution of ORF2 protein was visualized equally well with monoclonal and polyclonal α-ORF2 antibodies. Since ORF3 protein can react with ORF2 protein in some circumstances (17), it was of interest to determine whether ORF2 protein would form these granules in the absence of ORF3 protein. In all experiments, the efficiency of transfection was ca. 10%. Of the 10% of the transfected HuH-7 cells containing detectable ORF2 antigen on day 14 posttransfection, the subpopulation of these cells containing granules was similar (38 and 31%, respectively) in mutant compared to wild-type transfected cells: by day 21 the subpopulation of ORF2-positive cells containing ORF2 granules had doubled to ca. 74% (72 versus 76%) in both sets of cells.

Luciferase production from reading frame 3. The mutation that eliminated ORF3 protein production was predicted to act by prematurely terminating translation of ORF3. However, it was theoretically possible that a mutation at this site might prevent synthesis of the putative subgenomic RNA. To examine this possibility, we designed two new mutants that contained the luciferase gene in reading frame 3. The mutants were constructed by altering a functional replicon which synthesized luciferase as a reading frame 2 protein in place of viral ORF2 protein.

In the first mutant, the first 41 nt after the initiation codon for ORF3 were deleted, thus switching the luciferase gene from reading frame 2 to 3 and substituting it for the entire viral ORF3 gene. Luciferase activity was not detected in cells transfected with this mutant even though the genome encoded authentic luciferase (Fig. 4). This result was consistent with the previous results indicating that the sequence of the RNA at the beginning of ORF3 was required for expression of ORF3.

The second mutant was constructed from the original luciferase replicon by deleting the TG from the luciferase initiation codon thereby switching the gene from reading frame 2 into reading frame 3: this construct could potentially encode a

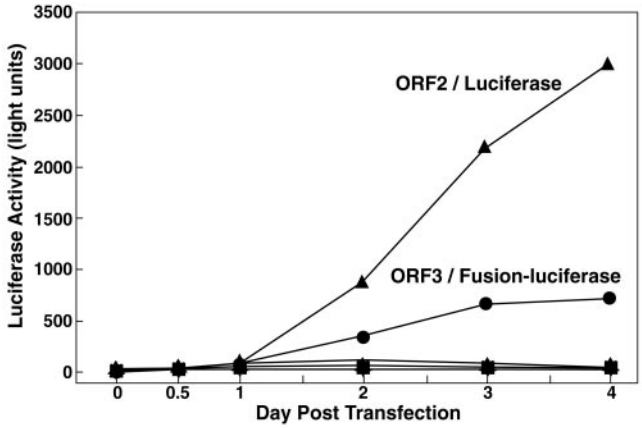


FIG. 4. An expressed ORF3 peptide-luciferase fusion protein exhibits less luciferase activity than does authentic luciferase expressed from reading frame 2. The luciferase activity detected in cells transfected with mutants expressing luciferase reading frame 2 protein (▲) or reading frame 3 fusion protein (●) is compared to that in mock-transfected cells (−), in cells transfected with the luciferase reading frame 2 mutant encoding an inactive RdRp (■), and in a luciferase reading frame 3 mutant in which the conserved nucleotide sequence was deleted (♦).

fusion protein beginning with the first 14 amino acids of viral ORF3 protein, followed by the luciferase sequence. This ORF3/fusion-luciferase mutant did produce functional luciferase in transfected HuH-7 cells, albeit at decreased levels compared to the control (Fig. 4). Nevertheless, these results suggested that the putative subgenomic RNA for reading frame 3 was transcribed and translated in this mutant.

ORF3 protein is required for infection of rhesus macaques. Since it was possible to delete 88% of the ORF3 protein coding sequence in the infectious clone and still produce ORF2 protein, this ORF3-null mutant could be tested for viability in vivo in order to determine whether ORF3 protein was required for viral infectivity. This would differ from the in vitro studies in that detectable infection of macaques requires not only RNA replication but also depends on the production of mature virus particles that are then able to infect enough additional liver cells to induce seroconversion. Capped transcripts synthesized in vitro from the ORF3-null mutant clone were inoculated intrahepatically into two rhesus macaques. An aliquot of the same transcription mixture was also transfected into HuH-7 cells, and cells were stained with anti-ORF2 and anti-ORF3 for immunofluorescence microscopy on day 8. As expected, ORF2 protein, but not ORF3 protein, was detected, indicating that the introduced termination codon was functional and that the viral genome was still replication competent (data not shown).

In contrast to what was seen in cell culture, the macaques did not display any indication of infection. Hepatitis, as determined by serum ALT levels, did not develop and neither animal (CJ7A and CJ7R) seroconverted to anti-HEV (Table 1). Normally, seroconversion occurs between 4 and 8 weeks posttransfection, but in order to ensure that a slow course of infection was not missed, the animals were monitored for 17 weeks. The animals were reinoculated 5 months later with a new batch of transcripts. Once again, neither animal showed any indication of infection and both remained seronegative for anti-HEV.

An ORF3 mutant with an altered phosphorylation site is infectious for rhesus macaques. Since ORF3 protein was required for infection of macaques and since phosphorylation of ORF3 protein at serine 80 is postulated to be important for protein function, we mutated serine 80 so that phosphorylation was prevented and sought to determine whether this mutant was viable in vivo. In the first case, the serine 80 codon TCA was mutated to GCA, a mutation that changed serine to alanine (S80A) in ORF3 protein but, because of overlapping reading frames, also changed valine 66 of ORF2 protein to glycine.

Transcripts from the S80A mutant were replication competent when transfected into HuH-7 cells since immune fluorescence microscopy demonstrated that cells were stained for both ORF2 and ORF3. Comparison by confocal microscopy of cells transfected with wild-type or the S80A mutant revealed a similar distribution of ORF2 and ORF3 proteins without detectable colocalization (data not shown). In contrast, aliquots of the same transcription reaction were not infectious when inoculated into two macaques: both animals (H666 and H667) remained seronegative for anti-HEV during the 16 weeks of follow-up (Table 1). The same animals, still anti-HEV negative, were reinoculated with new transcripts of S80A 3 weeks later. Whereas one animal remained seronegative, the second animal did seroconvert to anti-HEV, although the antibody titer was never higher than 1:100 and rapidly decreased to borderline positive (Table 1 and Fig. 5A and B). Unfortunately, the peak virus titer determined by real-time RT-PCR was only 1 genome per 100 μl of serum, and it was not possible to amplify specifically the ORF3 gene by RT-PCR despite multiple attempts.

Since the S80A mutant was not infectious or was, at best, extremely attenuated in vivo, it was important to determine whether removal of the phosphorylation site in ORF3 protein or the coordinate mutation at amino acid 66 of ORF2 protein was detrimental. Therefore, the TCA serine 80 codon of ORF3 was mutated to the TTA codon for leucine, a mutation which did not introduce any amino acid changes into ORF2 protein. This mutant genome, S80L, also replicated in HuH-7 cells and produced the expected pattern of ORF2 and ORF3 protein expression and distribution that was indistinguishable from wild-type and the S80A mutant when analyzed by confocal microscopy (data not shown). More importantly, however, transcripts from this same reaction mixture were infectious when transfected into the liver of macaques (Fig. 5C and D). Each of the two inoculated animals (H695 and H696) seroconverted to anti-HEV (peak titer of 1:10,000) at week 5 postinoculation and each was viremic for at least 3 weeks with peak virus titers that were 50- to 53-fold higher than that found previously with the S80A mutant. These virus titers were lower than those seen in animals previously transfected with wild-type virus (2), a result that could reflect a degree of attenuation or, alternatively, be due to the normal biological variation that is frequently observed in these animals (2). The region of the viral genome spanning ORF3 (nt 4903 to 5663) was amplified from serum collected from each animal at week 3. In both animals, the TTA codon for leucine was the only sequence found at the mutation site, indicating that phosphorylation of serine 80 was not required for intrahepatic viral genome replication in macaques or for production of virus that was infectious for other hepatocytes. In addition, there were not any new mutations in the ORF3 sequence recovered from either animal.

DISCUSSION

We constructed mutants of our infectious cDNA clone of HEV and determined their phenotype in macaques and/or in cell culture in an effort to determine whether ORF3 has a critical role in the virus replication cycle.

Genomic RNA replication, as deduced from studies of HEV replicons, does not require ORF2 or ORF3 proteins because these two genes are simultaneously deleted and severely truncated, respectively, in these replicons (1, 3). However, because ORF2 and ORF3 proteins interact with each other in plasmid-based expression systems (17), it seemed possible that expression of one in the absence of the other might have an observable effect on viral replication in cell culture. Therefore, replication-competent mutants incapable of expressing ORF2 or ORF3 genes but otherwise unimpaired were constructed. It should be noted here that, although we refer to the ORF3 mutant as a null mutant, this mutated gene could encode the N-terminal 15 amino acids of ORF3 protein and this peptide could conceivably perform some function of ORF3. However,

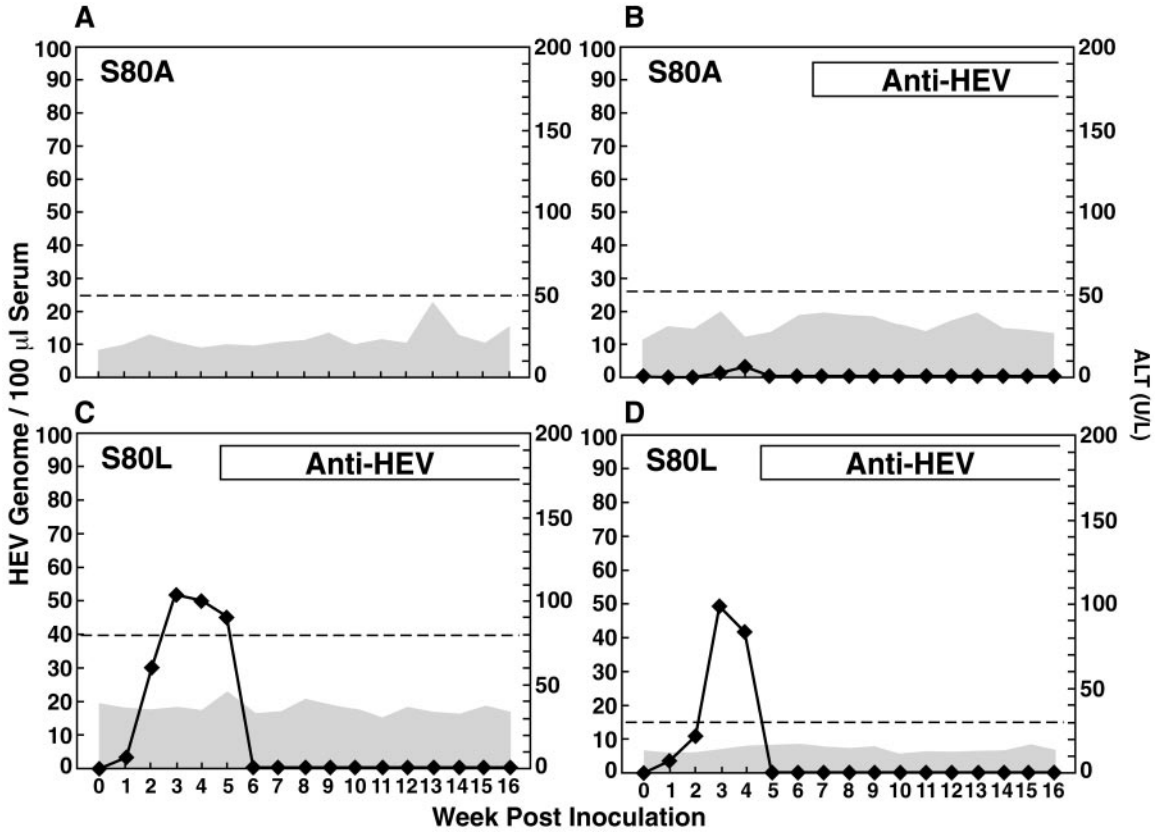


FIG. 5. Rhesus macaques transfected with the S80A mutant either did not get infected or experienced a highly attenuated infection compared to macaques transfected with the S80L mutant. Dotted line, ALT cutoff for hepatitis; ♦, genomes; shaded, ALT. Macaques were transfected intrahepatically with genomes containing serine 80 mutated to alanine (A and B) or to leucine (C and D).

since the region of ORF3 that interacts with ORF2 in vitro was mapped to amino acids 57 to 81 (17), we do not believe this peptide, even if stable, would influence our results.

Immune fluorescence microscopy demonstrated that ORF3, but not ORF2, protein was produced in cultured cells transfected with a mutant in which some ORF2 methionine codons were changed and translation-termination codons were introduced. Moreover, the distribution of ORF3 protein in these cells was indistinguishable from that in cells transfected with wild-type genomes that expressed both proteins. Therefore, genome-based expression of ORF3 protein without ORF2 protein did not result in a recognizable novel phenotype. Since this ORF2-null mutant would be unable to produce a capsid and hence could not spread, it was presumed to be noninfectious in vivo and was not tested in macaques.

It was much more difficult to construct a mutant virus that did not synthesize ORF3 protein but still made ORF2 protein. Synthesis of either protein depended on maintaining the RNA sequence immediately downstream of the ORF3 translation-initiation codon. This sequence (nt 5105 to 5116) consists of 12 nt that are universally conserved in all mammalian HEV genomes sequenced to date and are highly conserved in the very divergent avian HEV genome. Mutations in this region abolished synthesis of both ORF2 and ORF3 proteins even when the amino acid sequence should not have been affected. Therefore, the RNA sequence in this region functions as a CRE which is critical for some step in gene expression. Unfortunately, the inability to monitor viral RNA synthesis directly in this system made it impossible to determine whether full-length genome replication was impacted, but it would seem more likely that synthesis or translation of subgenomic RNAs thought to encode ORF2 and ORF3 proteins would be affected since this CRE is close to the 5′ end of both of these genes. Further mutational analyses are required to determine whether any other highly conserved nucleotides in the surrounding region contribute to the CRE function.

The absolute necessity of the CRE for ORF3 expression was confirmed when attempts were made to express luciferase from ORF3. Luciferase activity was not detectable if the entire ORF3 coding sequence, including the conserved sequence, was replaced with that encoding luciferase (Fig. 4). However, luciferase was produced if the conserved sequence was maintained and the luciferase gene was fused to the ORF3 gene 39 nt downstream of the first methionine codon in ORF3. The level of luciferase activity was well above background but was considerably less than that observed when luciferase was expressed from the same region of the genome as a reading frame 2 protein (ORF2/Luciferase). The lower level of luciferase activity when expressed from ORF3 might reflect differences in subgenomic RNA quantities or in translation efficiency or it simply could be due to reduced enzymatic activity because of fusion with ORF3 peptide.

Elimination of ORF3 protein synthesis did not affect the production or distribution of ORF2 protein in cell culture, suggesting that genomic and subgenomic RNA replication was normal. The replicon data combined with the mutant data suggested that neither ORF2 nor ORF3 protein, either alone or in concert, have a measurable affect on viral RNA synthesis. However, the lack of ORF3 protein could affect encapsidation of the RNA, capsid assembly or virion maturation necessary for viral infection. Therefore, it was cogent to ask whether a virus mutant unable to synthesize ORF3 protein was still infectious for macaques.

ORF3 protein is required for the infection of macaques. There was not any indication of HEV infection after four attempts to transfect macaques with an ORF3-null mutant containing synonymous mutations and two attempts with a frameshifted mutant. Therefore, ORF3 protein appears to be necessary for the virus to spread within the liver.

Phosphorylated ORF3 protein has been shown to interact with ORF2 protein in the yeast two-hybrid system, in COS-1 cells transiently transfected with plasmids expressing each protein, and in coupled transcription-translation assays (17). These data were interpreted as evidence that ORF3 protein might have a well-regulated role in HEV capsid assembly.

The fact that the ORF3-null mutant could not infect macaques is consistent with its suggested role in capsid assembly, but this explanation falls short in that the interaction of ORF2 and ORF3 proteins observed in vitro absolutely required the phosphorylation of ORF3 protein at serine 80. When serine 80 was mutated to alanine to prevent phosphorylation, the two proteins no longer interacted in the yeast two-hybrid system (17). In agreement with those findings, we were unable to infect three of four macaques with wild-type genomes in which serine 80 was mutated to alanine: the fourth macaque in the present study did minimally seroconvert to anti-HEV, suggesting limited viral replication had occurred, but the infection was so attenuated that we were unable to recover ORF3 viral sequence and so could not determine whether the mutation at amino acid 80 in ORF3 and the coincident valine to glycine change at amino acid 66 in ORF2 were maintained. In contrast, an alternative mutant in which phosphorylation was prevented by mutating serine 80 to leucine instead to alanine infected both macaques into which it was inoculated (Fig. 5). In this case, the ORF3 region was amplified from the serum of both macaques at the time of peak viremia and was confirmed to have retained the leucine mutation. Therefore, phosphorylation of serine 80 of ORF3 protein was not necessary for infection in vivo.

The reason for the inability of the ORF3 alanine mutant to infect macaques has not been identified. Since the ORF3 mutant contained an additional mutation in ORF2, this second mutation could have been responsible. However, it cannot be ruled out at this time that the alanine substitution itself was detrimental to ORF3 function and prevented both infection in vivo and ORF2/ORF3 interaction in vitro. This question might best be answered by determining whether a leucine instead of an alanine substitution would permit the two proteins to interact in the yeast two-hybrid system.

Although the infection course of the serine 80 to leucine mutant was similar to that we have observed previously in wild-type HEV transfections (2), especially as far as timing of viremia and seroconversion, the levels of viremia were on the low side in both animals. Thus, although the experiments demonstrated that phosphorylation was not crucial, it may be premature to conclude it is not beneficial in a natural infection that includes possible extrahepatic replication sites.

In summary, we have identified a CRE that is located in the ORF3 gene and plays a role in expression of both ORF2 and ORF3 proteins. Neither of these proteins is required for replication of the genome in cell culture but ORF3 protein (and presumably ORF2 protein) is absolutely required for infection in vivo. ORF3 protein may function in capsid assembly as previously suggested but, if it does, it need not be phosphorylated.

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