Mutations within Potential Glycosylation Sites in the Capsid Protein of Hepatitis E Virus Prevent the Formation of Infectious Virus Particles[▽]

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Hepatitis E virus is a nonenveloped RNA virus. However, the single capsid protein resembles a typical glycoprotein in that it contains a signal sequence and potential glycosylation sites that are utilized when recombinant capsid protein is overexpressed in cell culture. In order to determine whether these unexpected observations were biologically relevant or were artifacts of overexpression, we analyzed capsid protein produced during a normal viral replication cycle. In vitro transcripts from an infectious cDNA clone mutated to eliminate potential glycosylation sites were transfected into cultured Huh-7 cells and into the livers of rhesus macaques. The mutations did not detectably affect genome replication or capsid protein synthesis in cell culture. However, none of the mutants infected rhesus macaques. Velocity sedimentation analyses of transfected cell lysates revealed that mutation of the first two glycosylation sites prevented virion assembly, whereas mutation of the third site permitted particle formation and RNA encapsidation, but the particles were not infectious. However, conservative mutations that did not destroy glycosylation motifs also prevented infection. Overall, the data suggested that the mutations were lethal because they perturbed protein structure rather than because they eliminated glycosylation.

Hepatitis E virus (HEV) is transmitted via the fecal-oral route, predominantly through contaminated water. HEV causes acute self-limiting hepatitis and in much of the developing world is responsible for sporadic infections, as well as large epidemics, of acute hepatitis E, especially in Asia and Africa.

Four genotypes comprising a single serotype of mammalian HEV have been identified (26, 28). HEV was recently classified as the sole member of the genus *Hepevirus*, family *Hepeviridae* (3); sequence analysis suggests it is most closely related to rubella virus, an enveloped virus in the family *Togaviridae*. However, HEV does not contain a lipid envelope (26). The virion is 27 to 30 nm in diameter (1, 26) and has a sedimentation coefficient of 183S (2). The HEV genome is a positive-sense RNA, approximately 7.2 kb in length, with a short, capped 5' noncoding region and a 3' noncoding region preceding a poly(A) tail. Viral genomic RNA is infectious for some cultured cells and nonhuman primates: transfection with capped recombinant genomes results in production of infectious virions in vitro and acute hepatitis and/or seroconversion in vivo (6–8). The coding region of HEV contains three partially overlapping open reading frames (ORFs): ORF1 encodes the nonstructural proteins, ORF2 encodes the capsid protein, and ORF3, which overlaps the N terminus of ORF2, encodes a small protein of 114 amino acids (aa) (13, 15) that might be a regulatory protein (7, 19, 33, 44). The ORF2 and

ORF3 proteins are encoded by a bicistronic subgenomic RNA (13, 35, 45).

HEV does not grow sufficiently well either in cell culture or in nonhuman primates to permit direct biochemical analysis. Therefore, various in vitro systems have been utilized to characterize the viral proteins and their functions. ORF2 protein is the most thoroughly studied. Expression of ORF2 protein from a baculovirus vector in insect cells resulted in a protein that was truncated by 111 aa at its N terminus and by 53 aa at its C terminus to yield a 56-kDa protein containing 496 aa. This protein is immunogenic and induces neutralizing antibodies, but its relationship to authentic capsid protein is unknown (27, 30, 39, 40). Recombinant ORF2 protein binds to the 5' region of the viral genome (32), suggesting it may control RNA encapsidation. When expressed by itself in insect cells, ORF2 protein can assemble into virus-like particles (21, 22, 41, 47). Assembly is thought to involve dimer formation, and the site responsible for homo-oligomerization of recombinant ORF2 protein has been localized within its C terminus (20, 42, 46). The ORF2 protein sequence contains three potential sites for N-linked glycosylation, represented by the amino acid motif Asn-X-Ser/Thr, and a putative signal peptide sequence of about 15 aa at its N terminus. When overexpressed from a plasmid vector in mammalian cells, ORF2 protein clearly is glycosylated and transported to the cell surface (16, 37, 48). However, since the vast majority of nonenveloped viruses do not have glycoproteins, it is not clear whether these posttranslational modifications are biologically relevant. Characteristics described based solely on recombinant proteins overexpressed in isolation may differ from those present in the context of the normal viral replication cycle. This possibility prompted us to question whether the glycosylation of ORF2 protein observed

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under nonphysiological conditions actually has a biological function.

To explore the importance of glycosylation of ORF2 protein in the normal viral replication cycle, we eliminated the Asn within each of the glycosylation sequons, individually and in combination, by site-directed mutagenesis of an infectious cDNA clone and determined whether the capped RNA transcribed from each mutant genome was able to replicate in cell culture and/or was infectious for rhesus macaques. Since both ORF2 and ORF3 proteins are encoded by a subgenomic RNA (13), which must be synthesized *de novo* following infection or transfection, their detection in cells served as a convenient marker of genome replication (7), whereas seroconversion served as a marker for infection of nonhuman primates.

MATERIALS AND METHODS

Cells. S10-3 cells (5), a subclone of the human hepatoma cell line Huh-7 (23), were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 9% fetal bovine serum in a CO₂ incubator at 37°C. Transfected or infected cells were maintained in a CO₂ incubator at 34.5°C.

Construction of ORF2 mutants. The clone pSK-HEV2, hereafter referred to as pSar (8) (GenBank accession no. AAF444002), representing the consensus sequence of HEV strain Sar-55 (8, 38), served as the parental clone. An Asn-to-Gln substitution at each of the three putative glycosylation sites in ORF2 was introduced by site-directed mutagenesis of nucleotides AAC to CAA and fusion PCR into the parental clone. Plasmids pSar-G1, pSar-G2, and pSar-G3 contained the AAC-to-CAA change in ORF2 resulting in the single-amino-acid substitutions N137Q, N310Q, and N562Q, respectively. All three sites were mutated in pSar-G123. In plasmid pSar-G2(L₃₁₁A), CTC (the middle codon of the second glycosylation sequon) was mutated to GCC, resulting in the single-amino-acid substitution L311A, which should not affect glycosylation. Plasmid pSar-N₄₄₅Q contained an Asn-to-Gln substitution at amino acid 445, which is located in a highly conserved region that does not contain a glycosylation motif. Plasmids pSar-TSS(2) and pSar-TSS(4) were identical and contained a Ser-to-Thr substitution in glycosylation site 1 and Thr-to-Ser substitutions in glycosylation sites 2 and 3.

A fragment from nucleotides 6519 to 6965 of pSar and pSar-G3, respectively, was modified by PCR to include a 5' start codon and a 3' stop codon, inserted into the vector pGEM-T-Easy (Invitrogen), and cloned. These clones encoded wild-type ORF2 protein aa 459 to 607 of Sar55 (Sar/459-607) and a mutated ORF2 protein harboring the N562Q substitution (G3/459-607), respectively. Similar fragments (nucleotides 6516 to 6965) of pSar and pSar-G3, respectively, were cloned into the expression vector pRSET-C (Invitrogen) for generation of a polypeptide containing aa 458 to 607 of ORF2 protein with a hexamer-histidine tag and an Xpress epitope at the N terminus, as described previously (49).

Details of the cloning strategy and the oligonucleotides used to amplify fragments for cloning of each of the described constructs are available upon request. All plasmids were sequenced throughout the entire HEV protein to verify that unwanted mutations had not been introduced during the PCR steps.

Transcription in vitro and transfection of cultured cells. Plasmids were linearized at a unique BglII site located immediately downstream of the poly(A) tract of the HEV sequence. Capped transcripts were synthesized in a 100-μl volume with the T7 Riboprobe in vitro transcription system (Promega) in the presence of 0.5 mM cap analog 3'-O-methyl-m⁷G(5')pppG (Ambion), as described previously (12). For some applications, the transcription reaction was followed by treatment with RQ DNase I for 30 min at 37°C. The integrity and yields of the transcripts were determined by gel electrophoresis on a nondenaturing agarose gel.

S10-3 cells were transfected with DMRIE-C transfection reagent (Invitrogen) according to the manufacturer's instructions. For each experiment, S10-3 cells, grown to ~80% confluence, were washed twice with OptiMEM (Invitrogen) prior to addition of the transfection mixture. Cells in six-well plates were transfected with 900 μl/well OptiMEM containing 50 ng/μl DMRIE-C and 13.5 μl of the transcription mixture. Cells in T25 cell culture flasks were transfected with 2 ml OptiMEM containing 50 ng/μl DMRIE-C and 30 μl of the transcription mixture. The cells were incubated for 5 to 16 h at 34.5°C, after which the transfection mixture was exchanged for 2.5 ml (six-well plate) or 6 ml (T25 flask)

of Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 9% fetal bovine serum, and incubation was continued at 34.5°C.

Inoculation of rhesus macaques with HEV genomic RNA. Capped RNA was transcribed in vitro in a 200-μl volume from 10 μg BglII-linearized plasmid with the Promega T7 Riboprobe in vitro transcription system or, alternatively, with the T7 Megascript kit (Ambion), as described previously (8, 12). The integrity and yield of the transcribed RNA were verified by gel electrophoresis of a 5-μl aliquot of the reaction mixture. Another 5-μl aliquot was used to transfect S10-3 cells to verify the replication ability in vitro. The remaining 190 μl of transcription mixture was diluted with 810 μl of phosphate-buffered saline (PBS) without calcium and magnesium and immediately frozen on dry ice. Within 24 h, the mixture was thawed and injected into the liver of a rhesus macaque at multiple sites by percutaneous intrahepatic injection guided by ultrasound, as described previously (8). The macaques were prescreened for antibodies to HEV with a very sensitive enzyme-linked immunosorbent assay (9) in order to ensure that they would be susceptible to HEV infection. Following injection, sera were monitored weekly for serum alanine amino transferase levels (Anlytics, Gaithersburg, MD) and anti-HEV as described previously (8, 9). Each mutant was tested in two macaques simultaneously, and the animals were reinoculated with a second set of transcripts if they did not show signs of infection within 16 weeks.

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* (24).

Antibodies. Polyclonal anti-ORF2 was derived from a chimpanzee (chimp 1313) that had been sequentially inoculated with the human HEV strains Sar-55 and Mex-14 (6). The mouse monoclonal anti-ORF2, HEV no. 8, was a gift from GlaxoSmithKlein. The polyclonal anti-ORF3 was produced by Lofstrand in rabbits immunized with a synthetic peptide comprising ORF3 aa 91 to 123 of the human HEV strain Sar-55 (12). Mouse monoclonal antibody (MAb) against human golgin 97 was purchased from Molecular Probes. The secondary antibodies were matched to the species producing the primary antibody, except that anti-human immunoglobulin G (IgG) was used to detect chimpanzee antibodies. Alexa Fluor 488 goat anti-human IgG, Alexa Fluor 568 goat anti-rabbit IgG, and Alexa Fluor 568 goat anti-mouse IgG were all purchased from Molecular Probes; anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased from Jackson Immuno Research; and horseradish peroxidase-conjugated anti-hexamer-histidine antibody was purchased from Sigma.

Immunofluorescence microscopy. Transfected and mock-transfected S10-3 cells were trypsinized and replated in two-well glass chamber slides (Nalge Nunc, Inc.) and grown for an additional 2 days prior to being immunostained. The cells were washed briefly in PBS, fixed with acetone, and air dried. Following washing with PBS, the fixed cells were incubated with the primary antibodies at room temperature for 20 min and then washed with PBS and counterstained with the appropriate secondary antibodies. The slides were washed once more in PBS and covered with Vectashield mounting solution containing DAPI (4',6'-diamidino-2-phenylindole) (Vecta Laboratories). Samples were examined with a Zeiss Axioscope 2 Plus fluorescence photomicroscope or, alternatively, with a Leica SP5 confocal microscope (Leica Microsystems, Exton, PA) using a 63× oil immersion objective, numerical aperture 1.4, zoom 3. Fluorochromes were excited using a 405-nm diode laser for DAPI, a 488-nm laser for Alexa Fluor 488 (green), and a 568-nm laser for Alexa Fluor 568 (red). To avoid possible cross talk, the three wavelengths were collected separately and later merged. Confocal images were processed using Leica TCS-SP software (version 2.1537) and Huygens Essential software version 3.00 p5.

In vitro infectivity assay. Transfected S10-3 cells, verified by immunofluorescence microscopy to contain replicating HEV, were trypsinized and pelleted by centrifugation at low speed for 10 min. The pellet from cells in one well of a six-well plate was lysed by vortexing in 900 μl H₂O. After 10 min of incubation at room temperature, the sample was supplemented with 100 μl 10× PBS and centrifuged at 13,200 rpm for 2 min in a 5415C Eppendorf centrifuge to remove the cell debris. The lysate was used to infect cell monolayers as described previously (7). The infected cells were incubated at 34.5°C for 5 to 6 days, after which they were examined by immunofluorescence microscopy, and the number of cells containing ORF2 protein or ORF3 protein per well of an eight-well chamber slide (Nalge Nunc, Int.) was determined. Analysis was performed blinded and on duplicate or triplicate samples.

Velocity sedimentation gradient. S10-3 cells, grown to subconfluency in a T25 cell culture flask, were transfected with DNase-treated transfection mixture as described above. The cells were split 1:2 2 days posttransfection. One half of the cells were analyzed by immunofluorescence microscopy to monitor viral replication. The other half were cultured in a T25 flask for four more days, after which the cells were trypsinized, divided into two 2-ml Sarstedt tubes, and centrifuged

at low speed for 5 min. The cell pellet was lysed as described for the infectivity assay, treated with 250 Kunitz units of bovine RNase A (Sigma) and 5 μ l of 25% (vol/vol) NP-40 substitute (Fluka), layered onto a sucrose gradient (5 to 30% sucrose in PBS), and centrifuged for 65 min at 30,000 rpm at 20°C in an SW50.1 Beckmann rotor as described previously (7). Ten-drop fractions were collected from the bottom to the top of the gradient, and RNA was extracted from 100 μ l of each fraction with Trizol LS reagent (Invitrogen) as recommended by the manufacturer. The extracted RNA was quantified by real-time reverse transcription (RT)-PCR specific for HEV RNA as described previously (8).

Protein preparation and analysis. Transfected and mock-transfected S10-3 cells were trypsinized and centrifuged at low speed for 5 min. The cell pellet from one well of a six-well plate was resuspended in 100 μ l sodium dodecyl sulfate (SDS) gel-loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.5% beta-mercaptoethanol) containing proteinase inhibitor mixture (Complete; Roche). Samples were sonicated on ice three times for 1 min each time. The cell lysates were diluted in NuPAGE LDS sample buffer and reducing agent (Invitrogen), denatured for 10 min at 90°C, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a 4 to 12% NuPAGE Bis-Tris Gel (Invitrogen). The separated proteins were electrophoretically transferred onto a nitrocellulose membrane (0.45 μ m); blocked with StartingBlock blocking buffer (Pierce) containing 0.5% Tween 20; incubated with anti-ORF2 MAb HEV no. 8 (1:5,000) for 1 h at room temperature, followed by an anti-mouse horseradish peroxidase-conjugated secondary antibody (1:50,000) (Jackson Immuno Research); and visualized with a Visualizer Western blot detection kit (Upstate, Lake Placid, NY) as specified by the manufacturer.

His-tagged ORF2 polypeptides were expressed in *Escherichia coli* and purified by nickel-chelate affinity chromatography (Invitrogen) under denaturing conditions as previously reported (49). The purified polypeptides were gradually renatured by dialyzing them against 1 $\times$ PBS containing decreasing amounts of urea (4 M, 2 M, and 0.5 M) and finally dialyzed four times against 1 $\times$ PBS, pH 8.6 to 9.0. The concentration of each purified polypeptide was determined with the Micro BCA Protein Assay Reagent Kit (Pierce) according to the manufacturer's protocol. About 300 ng of each purified polypeptide was separated by SDS-PAGE on a Novex 14% Tris-glycine gel (Invitrogen), and protein bands were visualized with SimplyBlue SafeStain (Sigma) as recommended by the manufacturer. Alternatively, Western blot analysis was performed after electrophoretic transfer of the proteins to a nitrocellulose membrane. The membrane was blocked in 1 $\times$ PBS containing 0.05% Tween 20 and 5% skim milk powder, followed by incubation with horseradish peroxidase-conjugated anti-hexamethistidine (1:5,000; Sigma) for 1 h. After extensive washes, the protein bands were visualized using Sigma Fast (Sigma) as indicated in the manufacturer's instructions.

³⁵S-labeled ORF2 polypeptides (aa 459 to 607), synthesized in the TNT Coupled Reticulocyte Lysate System (Promega) with SP6 polymerase and [³⁵S]methionine, were mixed with an equal volume of 2 $\times$ Laemmli buffer (4% SDS, 10% mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8). One half of the reaction mixture was heated to 90°C for 5 min and electrophoresed on a Novex 14% Tris-glycine gel (Invitrogen). The other half was directly electrophoresed without being heated. The ³⁵S-labeled peptides were detected by autoradiography.

RESULTS

Glycosylation sequons are highly conserved in the capsid protein of mammalian HEV strains. In a first attempt, we wanted to evaluate the importance of glycosylation sequons in the HEV capsid protein by comparison of the amino acid sequences among the mammalian HEV strains present in GenBank. Seventy-four of the GenBank entries contained nearly complete ORF2 sequences. Translation of the nucleotide sequence and comparison of these mammalian strains showed that the three potential glycosylation motifs, ¹³⁷NLS (1), ³¹⁰NLT (2), and ⁵⁶²NTT (3), are highly conserved. Only three strains (GenBank accession numbers DQ279091, AY723745, and AB189070) contained an amino acid change within one of the three motifs, and only two of them contained a change that would prevent N-linked glycosylation at one site. The swine HEV strain DQ (DQ279091) contained the amino acid sequence ¹³⁷TLS instead of the glycosylation sequon

FIG. 1. Replication of HEV demonstrated by indirect immune fluorescence microscopy. S10-3 cells were transfected with capped in vitro transcripts of the parental construct pSar (Sar) and the mutants pSar-G1 (G1), pSar-G2 (G2), pSar-G3 (G3), pSar-G123 (G123), and pSar-G2(L₃₁₁A) (G2L₃₁₁A), respectively, and stained 5 days posttransfection for ORF2 protein (green) with chimp 1313 antiserum and Alexa Fluor 488 goat anti-human IgG. Nuclei were counterstained with DAPI.

¹³⁷NLS, and the other strain (AB189070) (34) contained the amino acid sequence ⁵⁶²DTT instead of ⁵⁶²NTT. However, examination of the complete sequence of the ORF2 protein showed that the high degree of amino acid sequence similarity was not limited to the potential glycosylation motifs but that regions of 100% identity among even the most genetically diverse strains could also be found throughout the protein (e.g., aa 176 to 187, aa 247 to 257, and aa 441 to 462). These other highly conserved regions did not contain recognized motifs.

HEV genomes with mutated glycosylation motifs replicate in vitro. In order to determine the possible requirement for glycosylation of ORF2 protein in a normal infection, we mutated the full-length infectious cDNA clone of genotype 1 strain Sar-55 to generate an Asn-to-Gln substitution within each glycosylation sequon individually or at all three sequons combined. Each mutation would prevent glycosylation of that site. We also attempted to determine the sequence plasticity of this region by introducing a Leu-to-Ala substitution at amino acid 311 at the second position within the second glycosylation motif: this substitution should not abolish glycosylation at the site but would alter the primary amino acid sequence. Capped transcripts synthesized in vitro were transfected into S10-3 cells and analyzed for successful virus replication by indirect immunofluorescence microscopy of ORF2 protein stained with chimp 1313 serum (6). As shown in Fig. 1, ORF2 protein was readily detectable in cells transfected with the wild-type Sar, with each of the single mutants, or with the triple mutant. ORF3 protein expression, another marker of genome replication, was also detected in all cases (data not shown). These findings suggested that HEV genomes replicated in these cul-

FIG. 2. Confocal immune fluorescence microscopy of S10-3 cells transfected 6 days earlier with Sar and G123 transcripts or mock transfected. Merged images of representative transfections are presented. (A) ORF2 protein (green) and ORF3 protein (red). (B) ORF2 protein (green) and the Golgi apparatus (red, anti-golgin 97). (C) ORF2 protein (green) and the ER (red, anti-calnexin). Mock-transfected cells, stained with the same antibodies in each experiment, are shown for comparison.

tured cells as expected and that ORF2 protein containing recognizable epitopes was synthesized in apparently normal amounts.

Interestingly, at the time of analysis (5 days posttransfection), most, although not all, mutant G2- and G123-transfected cells displayed accumulation of ORF2 protein-positive granules in the cytoplasm, whereas ORF2 protein staining was more evenly distributed in most cells transfected with the wild type or the other mutants. Cells transfected with wild-type genomes sometimes contained similar granules at later times posttransfection, but there remained a clear difference in granule abundance between the wild type and mutants containing the N310Q mutation even at 10 days posttransfection. The basis of granule formation is unclear. The reported interaction of recombinant ORF2 protein and ORF3 protein (43) prompted us to determine if ORF3 protein was involved in granule formation. Examination by confocal microscopy of transfected cells stained for both ORF2 and ORF3 proteins

(Fig. 2A) showed no clear indication of colocalization of ORF2 protein and ORF3 protein in cells transfected either with Sar or with G123 (Fig. 2A). Similar results were obtained from samples analyzed 4, 6, or 10 days posttransfection (data not shown). Since glycoproteins are transported through the Golgi apparatus or endoplasmic reticulum (ER), we also investigated if ORF2 protein produced by replication of either the wild type or the G123 mutant associated with these organelles. Double labeling with anti-ORF2 protein and Golgi-specific anti-golgin-97 MAb (Molecular Probes) (Fig. 2B) or with anti-ORF2 protein and ER-specific anti-calnexin MAb (Affinity Bio-Reagents) (Fig. 2C) failed to reveal colocalization of ORF2 proteins with either organelle, whether in Sar-transfected cells or in G123-transfected cells.

The majority of ORF2 protein in S10-3 cells is not glycosylated. Western blot analysis of cell lysate harvested 6 days after transfection with Sar and G123 was performed to determine the glycosylation status of ORF2 protein. The molecular

FIG. 3. Wild-type Sar and mutant G123 transcripts produce ORF2 proteins of the same approximate size. Cell lysates of transfected and mock-transfected cells were separated by SDS-PAGE on a 4% to 12% Bis-Tris gel and immunoblotted with anti-ORF2 mouse MAb HEV no. 8. The molecular masses of protein markers (SeeBlue; Invitrogen) are indicated.

weight of overexpressed ORF2 protein is increased by glycosylation and decreased by variable truncation at the N terminus, C terminus, or both. Since these two modifications have opposite effects and it is not known if ORF2 protein is truncated when expressed normally, it is not possible to predict a reliable molecular weight for a glycosylated ORF2 protein. Therefore, since comparable amounts of glycosylated and nonglycosylated ORF2 proteins were observed under conditions of overexpression (31, 48), the wild-type and G123 mutant proteins were compared directly for any differences in migration rate or in number of bands. Comparison of electrophoretic mobilities suggested the ORF2 proteins were of identical masses (~70 kDa) when encoded by either wild-type Sar or the mutant G123 (Fig. 3). In contrast to previous reports describing the different electrophoretic migration rates of glycosylated and nonglycosylated recombinant ORF2 proteins highly expressed in COS-1 and BHK-21 cells (16, 37, 48), different glycosylated forms of the wild-type ORF2 protein were not detected in the viral replication system. Although these results cannot rule out addition of short oligosaccharides, they do suggest that at least the majority, if not all, of the ORF2 protein accumulating within transfected S10-3 cells is not glycosylated. This result is in agreement with a report by Torresi

et al. (36) that the nonglycosylated ORF2 protein is the stable form in mammalian cells.

Mutations within any individual glycosylation sequon are lethal. In order to determine if the replication-competent mutants produced infectious virus in S10-3 cells, we applied a newly established in vitro infectivity assay (5). Cell lysates were prepared from wild-type- or mutant-transfected S10-3 cells and tested for the ability to infect naïve cultured cells. As summarized in Table 1, the lysate from cells transfected with the wild-type Sar consistently contained infectious virions, as determined by indirect immunofluorescence microscopy performed 6 days postinfection. In contrast, none of the lysates from cells transfected with HEV mutant G1, G2, G3, or G123 were able to infect new cells, suggesting they did not contain infectious virions. In order to distinguish the importance of N-linked glycosylation versus conserved amino acid sequences for infectivity of HEV, we also tested cell lysates of S10-3 cells transfected with the G2(L₃₁₁A) mutant. This mutation should not prevent glycosylation but is in the middle of the ³¹⁰NLT glycosylation site identified previously as the major site for N-linked glycosylation (48). Lysates from cells transfected with this mutant failed to infect cultured cells in experiments in which infectious wild-type HEV was detected (Table 1, experiments 3 and 4). These data suggested that the amino acid sequence per se was very important in this region, irrespective of glycosylation.

Glycosylation-independent mutations that are lethal. Two classes of mutants in which glycosylation should not be affected were constructed to further define the impact of the amino acid sequence on infectivity. In the first case, Asn₄₄₅ (underlined in the ORF2 sequence QDYDNQH) was mutated to Gln (N₄₄₅Q) to mimic the mutations used to destroy the glycosylation motif in the mutants described in Table 1. This particular asparagine residue was chosen because it was within a highly conserved sequence, but in contrast to the mutations listed in Table 1, it was neither part of nor even near a glycosylation motif. Immunofluorescence staining of transfected cultures confirmed that similar numbers of cells had been transfected in each case. However, only the wild-type Sar produced virus, demonstrating that this apparently minor mutation completely prevented infection (Table 2).

TABLE 1. Infectivity of wild-type HEV and mutants

Construct	Infection in vitro (no. of infected cell foci in expt no.) ^a :				Infection in vivo (peak reciprocal α-HEV titer) ^b			
					First transfection		Repeat transfection ^c	
	1	2	3	4	A	B	A	B
Sar	24/21	60/50	22/20	294/225	10 ⁴	10 ⁴	ND ^d	ND ^d
G1	0/0	0/0	ND ^d	ND	<10 ²	<10 ²	<10 ²	<10 ²
G2	0/0	0/0	ND	ND	<10 ²	<10 ²	<10 ²	<10 ²
G3	0/0	0/0	ND	ND	<10 ²	<10 ²	<10 ²	<10 ²
G123	0/0	0/0	ND	ND	<10 ²	<10 ²	<10 ²	<10 ²
G2L ₃₁₁ A	ND	ND	0/0	0/0	ND	ND	ND	ND

^a S10-3 cell cultures were inoculated with equal amounts of lysate of transfected cells containing replicating HEV RNA (Fig. 1) and analyzed under code by indirect immunofluorescence microscopy with ORF2 protein- and ORF3 protein-specific antibodies. Duplicate wells were infected with the same lysate. The numbers of infected foci in duplicate wells of an eight-well chamber slide are given.

^b Rhesus macaques were inoculated with wild-type or mutant HEV RNA and monitored weekly for seroconversion over a period of 16 weeks. A peak reciprocal anti-HEV titer of >10² indicated seroconversion. Two individual animals (A and B) were inoculated with the same transcript.

^c The experiment was repeated by inoculating the same animals a second time.

^d ND, not done.

TABLE 2. In vitro infectivity assays of mutants with conservative changes

Construct	No. of infected cell foci ^a	
	Expt 1	Expt 2
Sar	414/420/501	41/37/24
N ₄₄₅ Q ^b	0/0/0	0/0/0
TSS (2) ^c	0/0/0	0/0/0
TSS (4) ^c	0/0/0	0/0/0

^a Same protocol as in Table 1, note a, except triplicate samples were tested.

^b N₄₄₅, which was not part of a glycosylation motif, was mutated to Q.

^c S was mutated to T or T was mutated to S in all three potential glycosylation sites; 2 and 4 are two different cDNA clones.

In the second class of mutants (TSS), the Ser or Thr in each glycosylation motif was mutated to Thr or Ser, respectively, thus introducing only a minor change in amino acid sequence while preserving each of the three potential glycosylation sites. Two independently derived clones of this mutant were tested, and neither mutant was able to infect a single cell, whereas the wild-type virus, prepared and tested in parallel in two independent experiments, was infectious and, in one case, was able to produce over 400 foci. These data confirmed that even a slight change in amino acid sequence could have a profound negative impact on the ability to produce infectious virus.

Mutation of any glycosylation sequon prevents infection of macaques. The cell culture system, which supports viral replication, is a useful tool to study HEV. However, the viral replication cycle in cultured cells may not completely mimic that in animals. To determine the infection competence of the HEV mutants *in vivo*, we inoculated capped transcripts of the ORF2-mutated constructs and of the wild-type Sar into the livers of rhesus macaques. The animals were monitored weekly until week 16. The two animals inoculated with the wild-type genomes seroconverted to anti-HEV 4 weeks after inoculation. In contrast, all of the mutants (G1, G2, G3, and G123) failed to infect animals, as evidenced by a failure of the animals to seroconvert to anti-HEV even after a second inoculation (Table 1). Therefore, substitution of Gln for Asn in any of the three conserved motifs eliminated the ability of the mutant to cause seroconversion in rhesus macaques, most likely because the virus was unable to spread cell to cell. These data correlated perfectly with the data obtained from the *in vitro* infectivity assays and suggest either that production of infectious virions requires glycosylated ORF2 protein at some stage or that the conserved amino acid sequence itself is required for proper formation or maturation of viral particles.

Unfortunately, direct determination of the glycosylation status of infectious wild-type HEV did not succeed due to the insufficient level of replication occurring in the available systems, and thus far, it has not been possible to purify sufficient virus from infected animals for biochemical analysis.

Mutation of either of two glycosylation motifs in ORF2 protein prevents particle formation. One logical explanation for the lack of infectivity of the investigated mutants might be that the mutations inhibited or prevented virion assembly. Therefore, velocity sedimentation gradient analysis was performed to determine if the mutant capsid protein assembled into virus particles and, if so, how their sedimentation rate compared to that of the wild type. Cell extracts of the various

transfected cells were treated with RNase A to eliminate or decrease the excess of unpackaged viral RNA (7) and then fractionated by velocity sedimentation on individual sucrose gradients. Real-time RT-PCR of samples taken prior to RNase digestion confirmed that similar amounts of HEV RNA were originally present in each lysate within an experiment (data not shown). Fractions were collected from the bottom to the top of the gradient, and total RNA was extracted and assessed by HEV-specific real-time RT-PCR. Figure 4 shows results from a representative experiment. In all gradients, some RNA was found at the top of the gradient, probably representing incompletely degraded viral RNA. Encapsidated wild-type Sar RNA sedimented near the bottom of the gradient with the peak amount in fraction 3. The sedimentation of the cell culture-derived wild-type particle was comparable to the sedimentation of RNase-treated HEV present in a stool specimen from an HEV-positive rhesus macaque (reference 7 and data not shown). In contrast, not even a hint of a peak was detected in this region in any of the gradients loaded with lysates containing either G1, G2, or G123, in which Asn had been replaced with Gln. The same results were obtained in an independently repeated experiment. Importantly, RNA also was not detected in this region of the gradient following fractionation of the lysate containing the G2(L₃₁₁A) mutant. G3 was the only mutant in which RNA was detected in the same sedimentation range as that of the wild type, suggesting that the mutant viral genome was encapsidated into a particle of approximately the same size as the wild-type virion. Differences in peak amounts of encapsidated Sar or G3 RNA varied in repeated experiments (Fig. 4 A, B, C, and D), suggesting Sar and G3 replicated and produced virus particles with comparable efficiencies. However, the G3 mutant was unable to infect either cultured liver cells or the livers of rhesus macaques. In sum, these data suggested that mutations at aa 137, 310, and 311 in ORF2 protein inhibited or prevented viral capsid assembly, and this explained their lethality, whereas the substitution at Asn 562 permitted capsid protein assembly into a particle, but for some reason, this particle was not infectious.

N562Q substitution affects dimerization of ORF2 polypeptide. It has been reported that part of the C-terminal region of the ORF2 protein encompassing aa 585 to 606 is critical for its dimerization (20, 46) (Y.-H. Zhou, unpublished data). This noncovalent dimerization of ORF2 proteins (16) is assumed to be important for the biogenesis of the HEV capsid (47). To elucidate if the N562Q mutation might affect the dimerization of the ORF2 protein, we prepared ³⁵S-labeled ORF2 polypeptides (Sar/459-607 and G3/459-607) by translation *in vitro*. The oligopeptides were analyzed under mildly denaturing conditions of SDS-PAGE and under the more harshly denaturing conditions achieved by heating the samples prior to SDS-PAGE. Figure 5A shows that the wild-type polypeptide migrated differently depending on whether the samples were heated or not (lanes 1 and 2). While only one band was detected if the sample was denatured by heating, two protein bands were observed if heating was omitted. The Sar oligopeptide migrated as an ~18-kDa monomer and also as a homodimer approximately twice the mass of the monomer; therefore, the wild-type polypeptide formed a dimer that was relatively resistant to denaturation by mild treatment with SDS. In contrast, the

FIG. 4. Evaluation of virus assembly by sucrose gradient velocity sedimentation. (Bottom) RNase A-treated lysates from S10-3 cells transfected with the wild-type Sar or the indicated mutant, G1, G2, G3, G123, or G2L₃₁₁A, were analyzed on individual gradients in parallel. The number of genomes present in each sample prior to RNase treatment was determined by real-time RT-PCR and showed ratios of 1.27 (G1), 1.48 (G2), 1.19 (G2L₃₁₁A), 1.32 (G3), and 1.19 (G123) in comparison to Sar. Fractions, collected from the bottom to the top of the gradient, were extracted with Trizol LS reagent, and viral RNA was quantified by HEV-specific real-time RT-PCR. (A, B, C, and D) Additional examples of sucrose gradient velocity sedimentation of lysates from S10-3 cells transfected with the wild-type Sar (▲) or the G3 mutant (◆).

G3 mutant polypeptide did not show any signs of dimerization under similar conditions (lanes 3 and 4). Only one protein band, migrating as a monomer (~18 kDa), was detected regardless of whether the sample was heat denatured or not. Similarly, His-tagged wild-type Sar/458-607 and His-tagged mutant G3/458-607, expressed in *E. coli* and purified by nickel-chelate affinity chromatography, displayed the same difference regarding the ability to form homodimers under mildly denaturing conditions (Fig. 5B). The homodimer of Sar/458-607 appeared to be relatively stable in the presence of SDS in contrast to G3/458-607, which either did not dimerize or formed a very labile dimer. In order to ensure the specificity of the results, Western blot analysis was performed (Fig. 5C). These results confirmed that the N562Q substitution prevented stable dimer formation of ORF2 protein under conditions that did not inhibit the wild type.

DISCUSSION

HEV ORF2 protein containing N-linked oligosaccharides was originally identified following overexpression of recombinant ORF2 protein in cell culture (16, 37); however, in the absence of an efficient cell culture system for the actual virus, it has been difficult to determine the relevance of ORF2 protein glycosylation to the normal virus growth cycle. It is well established that glycosylated proteins are an essential component of enveloped viruses. Cotranslational modification of such proteins by the addition of N-linked oligosaccharides provides increased solubility and possible interaction with chaperons in the ER and thus supports the process of folding and stabilizes the native conformation. Proteins of this type may aggregate irreversibly or exit the ER without assembling into oligomers if glycosylation is inhibited (14). N-linked glycosylation occurs on an Asn residue in the sequence Asn-X-Ser/Thr. However, the

FIG. 5. Dimerization of ORF2 polypeptides. (A) Autoradiography of ^{35}S -labeled ORF2 polypeptides, Sar/459-607 (Sar) and G3/459-607 (G3), synthesized *in vitro*. The polypeptides were mixed with Laemmli buffer, heated at 90°C for 5 min (H) or kept at room temperature (UH), and separated on a 14% Tris-glycine gel. (B) Purified His-tagged ORF2 polypeptides, Sar/458-607 (Sar) and G3/458-607 (G3), synthesized in *E. coli* were treated as described for panel A and visualized with SimplyBlue Safe Stain (Invitrogen). (C) His-tagged ORF2 polypeptides, Sar/458-607 (Sar) and G3/458-607 (G3), synthesized in *E. coli* were treated as described for panel A and analyzed by Western blotting with anti-hexamer-histidine antibody. The molecular masses of the protein markers are indicated in kDa. Note that the ORF2 polypeptides in panels B and C are larger than those in A due to the N-terminal Xpress epitope and hexamer-histidine tag.

presence of this sequon does not always mean this site is glycosylated (11). In contrast, the vast majority of nonenveloped viruses do not contain glycoproteins. Rotavirus is a rare example of a nonenveloped virus that contains glycosylated proteins (10). It has been shown that assembly of rotavirus occurs in the rough ER (17, 18).

The mutagenesis studies reported here indicated that the ORF2 capsid protein, a protein with an amino acid sequence that is highly conserved among the different HEV genotypes, is extremely sensitive to amino acid substitutions within the three potential glycosylation sites: virion formation was prevented by mutating the residue Asn within two of the three glycosylation sequons, and mutation of Asn in the third sequon resulted in a noninfectious virion. These results suggested that glycosylation might be an integral feature of virion morphogenesis. However, this conclusion was complicated by the demonstration that a different mutation within the second sequon also prevented the formation of virus particles, even though the potential for glycosylation remained. Even more confounding was the demonstration that exchanging Thr for Ser or vice versa in the glycosylation sequons totally prevented infectivity. Since the glycosylation motif was preserved, this mutant provided the most compelling evidence that even small perturbations of the sequence in these regions could not be tolerated. These results raised the question of whether the N-linked glycosylation motifs are conserved because they are utilized for glycosylation or because the primary amino acid sequence in these regions is itself critical for virus morphogenesis.

In spite of repeated attempts, neither glycosylated protein nor sequestration of ORF2 protein in the Golgi apparatus or ER compartments associated with glycosylation was detected even with the wild-type protein. Previous assays of recombinant ORF2 protein expressed in COS-1 cells had suggested that the protein was translocated across the ER and that the glycosylated form accumulated intracellularly, as well as on the cell surface (48). Recent data from Surjit et al. demonstrated an association of overexpressed recombinant ORF2 protein with the retrotranslocation pathway (31). The findings indicated that localization in the ER and glycosylation of the ORF2 protein are necessary to allow the transport of viral protein into the cytoplasm. Inhibition of glycosylation prevented the release of ORF2 protein from the ER into the cytoplasm (31). In contrast, ORF2 protein was not detected in

the ER in cells transfected with genome-length HEV transcripts of the wild type or of the G123 mutant (Fig. 2C). One possible explanation for the discrepancy is that the previous results were an artifact of overexpression and that ORF2 protein normally is not glycosylated and does not enter the ER. Alternatively, transit of wild-type ORF2 protein through the ER in the absence of substantial accumulation would probably not be detected due to the inefficiency of virus replication and of the methods available to detect it. However, colocalization of ORF2 protein with ER proteins due to retention of unglycosylated G123 ORF2 protein in the ER was also not detected, even though according to the retrotranslocation model (31), all of it should have accumulated there (Fig. 2C). It should be emphasized that replacement of the Leu residue by Ala at position 311 in the second glycosylation sequon, identified previously as the major site of glycosylation (48), also inhibited particle formation and infectivity (Table 1), even though the potential for glycosylation remained. Most importantly, the total absence of infectious virus when Ser was exchanged for Thr and vice versa in the glycosylation sequons (Table 2) was compelling evidence that the sequence in these regions is conserved because of structural constraints unrelated to glycosylation. Studies by Li et al. (22) are particularly relevant in that they showed that virus-like particles were formed by an N-terminal truncated ORF2 protein even though glycosylation was prevented, since the truncation deleted the signal sequence required for translocation into the ER.

The almost universal conservation of the three sequons across the four mammalian genotypes of HEV suggests that the amino acid sequence in this region is important, but it does not prove that the sites are utilized for glycosylation. The amino acid sequence of the entire ORF2 protein varies by less than 10% across genotypes, and there are other short regions that are as highly conserved as the glycosylation sequons even though they lack recognizable motifs. It is important to note that changing an Asn to Gln in one of these conserved regions also eliminated infectivity (Table 2). The major neutralization epitopes of HEV are nonlinear and cross-reactive across genotypes, suggesting they too are highly conserved and that precise folding is probably required to present the tertiary structure required for viability (29). Indeed, the capsid assembly process is believed to require a dimerization step, which appeared to be affected by mutation of the third sequon (Fig.

5): although virus particles were still formed and viral RNA was incorporated in apparently normal amounts, the particles were not infectious, suggesting that the viral capsid is very sensitive to small changes in its amino acid sequence. Additionally, the fact that HEV is heat inactivated at 56°C, a temperature almost 10°C lower than that required to inactivate hepatitis A virus (4), suggests the HEV virion is not that stable to begin with.

In summary, the data supporting glycosylation of ORF2 protein are based on (i) demonstrating oligosaccharides attached to recombinant ORF2 protein that was expressed at abnormally high concentrations in the absence of other viral proteins, (ii) the conservation of 3-aa-long glycosylation motifs across the four mammalian genotypes of HEV, and (iii) the demonstration that the sequence of HEV is most closely related to that of rubella virus, which is enveloped and contains two glycoproteins (25). Against the conclusion that ORF2 protein is glycosylated are (i) the failure to detect a migration difference in SDS-PAGE of two physiologically expressed ORF2 proteins, one capable of being glycosylated at three sites and the other incapable of being glycosylated; (ii) the universal conservation of other regions lacking motifs but containing more amino acids and the demonstration that introduction of a mutation into one such region was lethal; (iii) the failure to detect either the wild-type or G123 mutant ORF2 protein in the ER or Golgi apparatus in the cell culture replication system; (iv) the lethality of an Asn-to-Gln mutation in any one of the three potential glycosylation sites; (v) the lethality of Ser-to-Thr or Thr-to-Ser mutations in the sequons; and (vi) the precedent of the extreme rarity of examples of a glycoprotein in a nonenveloped virus. Therefore, stable glycosylation of ORF2 protein produced under normal conditions appears unlikely. Transient glycosylation or addition of a minimal number of sugar residues cannot be ruled out at present due to the insensitivity of available systems. However, it is not clear what advantage such glycosylation would present.

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