The Phosphorylated Form of the ORF3 Protein of Hepatitis E Virus Interacts with Its Non-glycosylated Form of the Major Capsid Protein, ORF2

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Hepatitis E virus (HEV) is a human RNA virus containing three open reading frames. Of these, ORF1 encodes the viral nonstructural polyprotein; ORF2 encodes the major capsid protein, which exists in a glycosylated and non-glycosylated form; and ORF3 codes for a phosphoprotein of undefined function. Using fluorescence-based colocalization, yeast two-hybrid experiments, transiently transfected COS-1 cell co-immunoprecipitation, and cell-free coupled transcription-translation techniques, we have shown that the ORF3 protein interacts with the ORF2 protein. The domains involved in this ORF2-ORF3 association have been identified and mapped. Our deletion analysis showed that a 25-amino acid region (residues 57–81) of the ORF3 protein is required for this interaction. Using a Mexican HEV isolate, site-directed mutagenesis of ORF3, and a phosphatase digestion assay, we showed that the ORF2-ORF3 interaction is dependent upon the phosphorylation at Ser⁴⁰ of ORF3. Finally, using COS-1 cell immunoprecipitation experiments, we found that the phosphorylated ORF3 protein preferentially interacts with the non-glycosylated ORF2 protein. These findings were confirmed using tunicamycin inhibition, point mutants, and deletion mutants expressing only non-glycosylated ORF2. ORF3 maps in the structural region of the HEV genome and now interacts with the major capsid protein, ORF2, in a post-translational modification-dependent manner. Such an interaction of ORF2 with ORF3 suggests a possible well regulated role for ORF3 in HEV structural assembly.

Hepatitis E is an acute disease endemic in many countries throughout developing parts of the world, in particular on the continents of Africa and Asia, where it causes epidemics and sporadic infections. The causative agent, hepatitis E virus (HEV), is transmitted via the fecal-oral route, predominantly through contaminated water (1). HEV is an RNA virus with a positive-sense genome (7.2 kb in length with three open reading frames (ORF1, ORF2, and ORF3) encoding three different proteins (2–4). ORF1 (5079 bp) is at the 5' end of the genome and is predicted to code for putative nonstructural proteins with sequences homologous to those encoding viral methyltransferases, proteases, helicases, and RNA-dependent RNA polymerases (3–6). In the absence of a reliable in vitro culture system for HEV, fundamental studies on its replication and expression strategy have not been undertaken. ORF2 and ORF3 have been expressed in Escherichia coli, animal cells, baculovirus, and yeast and in vitro in a coupled transcription-translation system (7–11). ORF2 encodes the major HEV structural protein, which has been shown to be an 88-kDa glycoprotein that is expressed intracellularly as well as on the cell surface. It is synthesized as a precursor and is processed through signal sequence cleavage into the mature protein, which is capable of self-association (12, 13). When expressed through the baculoviral expression system, ORF2 was shown to assemble into virus-like particles (VLPs), which were cell-associated as well as secreted in the culture medium (14, 15).

ORF3 encodes a small 13.5-kDa phosphoprotein that is expressed intracellularly and shows no major processing. It associates with the cytoskeletal and membrane fractions of cells (16, 17). Recently, the ORF3 protein has been shown to dimerize in a yeast cellular environment, and its dimerization domain has been mapped to a 43-amino acid region overlapping the SH3 binding and phosphorylation domains (13). Furthermore, ORF3 has been recently shown to interact with SH3 domains and to activate MAPK (18).

Heterotypic interactions of the HEV proteins have not been studied as yet. Although ORF3 is located in the 3'-third of the genome and has been termed a structural protein, there is no evidence to date for its involvement in HEV structural assembly. Because ORF2 is the major capsid protein, we undertook studies to examine colocalization of ORF2 and ORF3 in infected cells and to test for heterotypic interactions between these two viral proteins to evaluate a structural role for ORF3.

In the few years since its introduction, the yeast two-hybrid system has proven invaluable for studying physical interactions between genetically defined partners, for identifying contacts among the subunits of multiprotein complexes (19–21), and for mapping specific domains involved in protein-protein interactions (22–24). In this system, two plasmid-borne gene fusions are cotransformed into yeast cells, and the interaction between these two fusion proteins is measured by the reconstitution of a functional transcriptional activator that triggers the expression of reporter genes lacZ and HIS3.

We have used the yeast two-hybrid system along with fluorescence-based colocalization experiments, transiently transfected COS-1 cell immunoprecipitation, and coupled transcription-translation techniques to show the interaction of ORF3
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with ORF2. We have further mapped the interaction domain of ORF3 to a 25-amino acid region. Within this region, we have shown that a single amino acid (Ser<sup>80</sup>) is responsible for this heterotypic protein-protein interaction. Ser<sup>80</sup> has been shown to be the site for phosphorylation of ORF3. Our yeast two-hybrid analysis using a Mexican HEV isolate, site-directed mutagenesis, and a phosphatase digestion assay revealed that the ORF3-ORF3 interaction is phosphorylation-dependent. Finally, using immunoprecipitation experiments, followed by tunicamycin inhibition, point mutations, and deletion mutations expressing only the non-glycosylated form of ORF2, we have shown that phosphorylated ORF3 preferentially interacts with non-glycosylated ORF2. A possible role of ORF3 in a post-translational modification-dependent interaction with ORF2 is discussed in light of our results.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Plasmid Constructs**—All strains, plasmids, and plasmid constructs used in this study are described in Table I. The full-length ORF2 and ORF3 genes of HEV were excised from the pMT-ORF2 and pSG-ORF3 vectors (9, 25), respectively, and cloned into the yeast two-hybrid vectors, resulting in an N-terminal in-frame fusion with either the GAL4 DNA-binding or activation domain. DNA manipulations were carried out as described by Sambrook et al. (26). All deletion constructs were generated by subcloning the full-length ORF2 and ORF3 genes of HEV and are described in Table I. Plasmid constructs not containing fully compatible ends were screened for the correct reading frame by sequencing, whereas all other constructs with fully compatible ends were verified by restriction digestion and sequencing.

**Immunofluorescence Analysis**—COS-1 cells were plated at a concentration of ~50% on coverslips 1 day before transfection and grown for 18 h. 40 h post-transfection, phosphate-buffered saline (PBS)-washed cells were fixed with 2% paraformaldehyde in PBS at room temperature for 10 min, and then washed with 100% methanol at –20 °C for 3 min. Cells were then rehydrated with PBS for 20 min at room temperature. The cells were blocked with 5% normal goat serum for 2 h at room temperature and then incubated with appropriately diluted primary antibodies in PBS and 0.5% Tween 20 (PBST) containing 1% normal goat serum for 2 h at room temperature. The primary antibodies used were mouse monoclonal anti-ORF3 antibody (1:200 to 1:500 dilution) and rabbit polyclonal anti-ORF2 antibody (1:100 to 1:200 dilution). Cells were then washed three times with PBST for 5 min each and then incubated for 1 h at room temperature with a 1:1000 dilution of conjugated secondary antibodies. For colocalization experiments, the secondary antibodies used were goat anti-mouse IgG coupled to Alexa 488 dye and goat anti-rabbit IgG coupled to Alexa 594 dye (Molecular Probes, Inc., Eugene, OR). These were chosen to label the ORF3 protein with Alexa 488 dye (100 µg of DNA), whereas all other constructs with fully compatible ends were verified by restriction digestion and sequencing.

**In Vitro Transcription-Translation Assay**—The full-length ORF2 protein (pRSET-ORF2, encoding 660 amino acids of ORF2 with an N-terminal His<sub>6</sub> tag) and a full-length ORF3 protein (ORF3, amino acids 1 to 123, along with its deletion mutation) were expressed in yeast two-hybrid vectors, resulting in an N-terminal in-frame fusion with an N-terminal His<sub>6</sub> tag. The His<sub>6</sub>-labeled ORF3 protein (full-length or deletion mutation) was then bound to Ni<sup>2+</sup>-Sepharose beads (Amersham Biosciences), and the mixture was incubated in 50 mM potassium bicarbonate, pH 8.0, 50% with 10 µl of the parent vector (pSGI) was used. For each test, the wild-type ORF3 protein was expressed in yeast genetic assay for reconfirming positive two-hybrid interactions (30, 31). Plasmid constructs were extracted from the positive Y190 transformants (BD-ORF3/AD-ORF2, clones 1 and 2). The plasmids isolated from these clones were separated and verified using <i>E. coli</i> HB101 cells on M9 synthetic medium lacking Leu. Subsequently, these plasmids were singly transformed into the PJ69-4a and PJ69-4a <i>Saccharomyces cerevisiae</i> diploid strains was tested by plating for growth on SD plates in the absence of His (SDHis<sup>−</sup>). All possible control transformations were conducted and were verified to be negative for His<sub>3</sub> prototrophy.

For each test, the phosho-Eagl modified Eagle’s medium containing 10% fetal bovine serum and 20 µg/ml gentamycin. Cells were transfected at a density of ~50% with plasmid DNA using Lipofectin (Invitrogen) according to the manufacturer’s guidelines. For each 80-mm diameter culture well, 4 µl of DNA was used. The <sup>35</sup>S-labeled ORF2 protein (full-length or deletion mutation) was then added to the same tube and incubated for 4 h at 4 °C with gentle shaking. The beads were washed three times with PBS, resuspended in 10 ml of SDS-PAGE loading buffer (50 mM Tris-HCl (pH 6.8), 5% mercaptoethanol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol), and boiled for 4 min to dissociate the bound proteins. Aliquots (10 µl) of the supernatants were subjected to SDS-PAGE, and the <sup>35</sup>S-labeled proteins were detected by autoradiography.
RESULTS

Colocalization of HEV Structural Proteins ORF2 and ORF3—Dual labeling immunofluorescence microscopy revealed colocalization of ORF3 and ORF2 in COS-1 cells transiently transfected with the expression vectors pMT-ORF3 and pMT-ORF2 (Fig. 1). The distribution of ORF3 in these cells was cytoplasmic and displayed punctate green staining (Fig. 1, ORF3–488). Distribution of ORF2 was observed in the cytoplasm, too, and was denser around the nucleus, possibly in the endoplasmic reticulum, and stained red (Fig. 1, ORF2–594). Both proteins colocalized in the cytoplasm and did not aggregate in the nucleus or other organelles as shown in yellow (Fig. 1, MERGE).

The Two Proteins ORF2 and ORF3 Interact with Each Other.—The full-length ORF2 gene was cloned into the yeast two-hybrid vector containing the GAL4 DNA-binding domain, resulting in the expression of a fusion protein with ORF2 fused to the C terminus of the GAL4 DNA-binding domain. The full-length ORF3 gene was cloned in-frame into the two-hybrid vector containing the GAL4 activation domain, resulting in the expression of a fusion protein with ORF3 fused to the GAL6-dependent HIS3 and lacZ expression as determined by growth on SDTrp-Leu-His− dropout medium and the blue color from filter-lift β-galactosidase assays, respectively (Fig. 2). The yeast extract/peptone/dextrose (YPD) plate (Fig. 2B) showed unrestricted growth of all transformants shown in the template (Fig. 2A). Neither plasmid alone induced HIS3 or lacZ expression in yeast. Single transformants, the host strain, and the BD-SNF1/AD-SNF4-positive control (27) were plated on all the restrictive media plates (Fig. 2C), whereas only transformants containing the AD plasmid or constructs derived from it grew on SDTrp plates (Fig. 2C), whereas only transformants containing the AD plasmid or constructs derived from it grew on SDTrp plates (Fig. 2D). Only the positive control (BD-SNF1/AD-SNF4) and the transformants containing both BD-ORF2 and AD-ORF3 were able to grow on SDTrp-Leu−His− plates (Fig. 2D).

Liquid β-galactosidase activity was determined for the positive clones along with all appropriate negative and positive controls using the substrate chlorophenol red β-n-galactopyranoside. The mean relative β-galactosidase activities are shown in Fig. 3A. The host strain (Y190) along with transformants with single plasmids (AD-ORF2 and BD-ORF3) showed negligible β-galactosidase activity. Cotransformants containing none or one of the two ORFs (BD/AD, BD/AD-ORF2, and BD-ORF3/AD) also showed negligible β-galactosidase activity; however, cotransformants containing AD-ORF2 and BD-ORF3 together showed a high β-galactosidase response.

We further investigated the level of activation of the HIS3 reporter genes for the full-length ORF2-ORF3 interaction in the presence of 50 μM 3-AT. Hundredfold serial dilutions of log-phase cultures of Y190 strains expressing BD-SNF1 and AD-SNF4 (BD-SNF1/AD-SNF4) and AD-ORF2 and BD-ORF3 (BD-ORF3/AD-ORF2) along with appropriate controls were plated on YPD, SDHis−, and SDHis− plus 50 μM 3-AT (Fig. 3B). These results indicate the strength of the protein-protein interactions as a function of His prototrophy. The BD-ORF3/AD-ORF2 cotransformants showed growth up to a 10−4-fold dilution on the SDHis− plus 50 μM 3-AT plate. This experiment showed that the ORF2-ORF3 interaction is strong and true.

The specificity of the ORF2-ORF3 interaction was also confirmed using a yeast genetic approach (30). After genetic crossing of the single transformants (haploids), the His3 prototrophy of the diploid strains (a/a) was tested. Only the diploids containing both BD-ORF3 and AD-ORF2 (BD-ORF3/AD-ORF2) showed a positive phenotype, similar to the positive diploid control (BD-SNF1/AD-SNF4) (Fig. 4). From all the above ex-
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Fig. 5. Two-hybrid results showing heterotypic interactions of the HEV proteins ORF2 and ORF3. A, template for B–F showing transformants streaked in each section of the plate; B–E, growth on YPD, SDTrp', SDLeu', and SDTrp-Leu'His' plates, respectively; F, results from the filter-lift β-galactosidase (β-Gal) assay. Growth is represented by white on a black background of the Petri dish. Blue is represented by the black streaks on a white background in the nitrocellulose membrane.

Table I

| Strain/plasmid/construct | Genotype/description/reference |
|--------------------------|--------------------------------|
| **Strains**              |                                |
| Y190                     | MATa trp1–901 his3 leu2–3,112 ura3–52 ade2 ga14 ga180URA3::GAL-lacZ LYS2::GAL-HIS3 |
| PJ69–4a                  | MATa trp1–901 leu2–3,112 his3–52 ura3–52 his3–200 ga14Δga180Δ Lye2::GAL-HIS3 GAL2-ADE2 met2::GAL7-lacZ |
| PJ69–4a                  | MATa trp1–901 leu2–3,112 ura3–52 his3–200 ga14Δga180Δ Lye2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ |

**Plasmids**

| pGAD424/pACT2 | GAL4 AD vector (GAL4 768–881); LEU2, 2μm, Amp' |
| pGBT9/pAS2 | GAL4 DNA-BD vector (GAL4 1–147); TRP1, 2μm, Amp' |
| pAS2-ORF3 | pSG-ORF3 digested with Smal and BamHI, fragment ligated |
| pGAD424/ORF3 | pMT-ORF2 digested with NcoI and BamHI, fragment ligated |
| pACT2-ORF2 | pGBT9-ORF3 digested with NcoI and BamHI, fragment ligated |
| pAS2-ORF3 | pSG-ORF3 digested with NcoI and BamHI, fragment ligated |
| pGAD424/ORF3-(1–57) | pGBT9-ORF3 digested with NcoI and BamHI, fragment ligated |
| pAS2-ORF3-(1–57) | pGBT9-ORF3 digested with NcoI and BamHI, fragment ligated |
| pGAD424/ORF3-(57–123) | pGBT9-ORF3 digested with NcoI and BamHI, fragment ligated |
| pAS2-ORF3-(57–123) | pGBT9-ORF3 digested with NcoI and BamHI, fragment ligated |
| pGAD424/ORF3-(57–81) | pGBT9-ORF3-(57–123) digested with EagI and EcoRI and religated |
| pAS2-ORF3-(57–81) | pGBT9-ORF3-(57–123) digested with EagI and EcoRI and religated |
| pGAD424/ORF3-(1–230) | pGBT9-ORF3-(57–123) digested with EagI and EcoRI and religated |
| pAS2-ORF2-(231–600) | pACT2-ORF2-(1–508) digested with HindIII and BamHI and religated |
| pGAD424/Orf3-(83–123) | pACT2-ORF2-(510–600) digested with HindIII and BamHI and religated |
| pAS2-ORF3-S80A | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pAS2-Orf3-Mex | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pMT-ORF3 | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pSG-ORF3(57–81) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pSG-ORF3(1–81) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pSG-ORF3(1–57) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3-(83–123) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3-(57–123) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3-(57–81) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3-(1–81) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3-(1–57) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3(57–81) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3(1–81) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |
| pGAD424/Orf3(1–57) | pAS2-ORF3 Mex digested with NcoI and EcoRI, fragment ligated |

Our experiments, we conclude that the two HEV proteins ORF2 and ORF3 interact with each other in a yeast two-hybrid system.

A 25-Amino Acid Region of the ORF3 Protein Binds to the Full-length ORF2 Protein.—To characterize the domains involved in the ORF2-ORF3 interaction, an array of deletion mutations were constructed for both ORF2 and ORF3 and were cloned into the yeast two-hybrid AD and BD vectors, as described in Table I. Combinations of full-length fusion constructs and deletion mutants of each fusion construct were tested for in vivo protein-protein interactions as shown in Fig. 5.

A phenomenon very clearly observed with the ORF2 protein was that none of its deletion mutants could interact with full-length ORF3 when tested in the yeast two-hybrid system. The ORF2 deletion mutants used in this study represented different parts of the protein and were from a variety of regions and lengths as shown in Fig. 5. On the other hand, ORF3 deletion mutants showed both positive and negative results with various different deletion mutants when tested with full-length ORF2 for two-hybrid interactions. The ORF3-(1–81) deletion fragment showed a positive interaction with full-length ORF2 upon the yeast two-hybrid assay. Consequently, when the ORF3-(83–123) deletion fragment was tested, it showed negative. These initial experiments indicated that amino acids 1–81 of the ORF3 protein contain the interaction domain. Furthermore, when the ORF3-(1–57) deletion fragment was tested...
with ORF2, the result was again negative, whereas ORF3-(57–81) showed a positive interaction with ORF2. All the above results indicate that the region between amino acids 57 and 81 of ORF3 contains the interaction domain. ORF3-(57–81) was constructed and tested for its interaction with ORF2. This interaction showed positive upon all yeast two-hybrid assays. From these experiments, we were able to clearly map a 25-amino acid region of the ORF3 protein that is responsible for its interaction with ORF2.

Ser80 Plays a Key Role in ORF2-ORF3 Interactions—The 25-amino acid interaction domain of ORF3 contains Ser80, which has earlier been shown by us to be the site for phosphorylation of this protein (17). An S80A point mutant of full-length ORF3 was cloned into the yeast two-hybrid BD vector. When full-length AD-ORF3 was tested with this BD-ORF3(S80A) point mutation, the results showed negative in the yeast two-hybrid analysis (Fig. 6). This result indicates that the ORF2-ORF3 interaction depends on the presence of Ser80.

The above experiments have clearly been able to pinpoint a single amino acid residue responsible for this protein-protein interaction. We designed a co-immunoprecipitation procedure to study the ORF2-ORF3 interaction and to validate our two-hybrid findings. Heterotypic interactions of the two HEV proteins ORF2 and ORF3 were studied by transiently transfecting COS-1 cells with either pMT-ORF2 alone (as a control) or in combination with one of the following: pMT-ORF3 (full-length, containing Ser80), pSG-BD-ORF3-(57–81) (containing the binding domain fused to the 25-amino acid interaction domain from ORF3), or pSG-ORF3(S80A) (full-length, containing an S80A point mutation) (Fig. 7). [35S]Methionine-labeled cell lysates were then immunoprecipitated with polyclonal antibodies. In a control experiment, the expression of both ORF2 and ORF3 was detected by their respective antibodies (Fig. 7, lanes 1–3). All binding studies were conducted with anti-ORF2 antibodies (Fig. 7, lanes 4–10).

Upon cotransfection of COS-1 cells with pMT-ORF3 and pMT-ORF2 constructs and immunoprecipitation with anti-ORF2 antibodies, both full-length proteins ORF2 and ORF3 were detected (Fig. 7, lane 10). In another set of cotransfection experiments, pSG-BD-ORF3-(57–81) and pMT-ORF2 were immunoprecipitated with anti-ORF2 antibodies, and bands corresponding to both ORF2 and BD-ORF3-(57–81) were detected (Fig. 7, lane 7), thus providing in vitro evidence that the 25-amino acid region (residues 57–81) contains the interaction domain. As a control, pSG-BD and pMT-ORF2 were immunoprecipitated with anti-ORF2 antibodies, which showed negative (Fig. 7, lane 8). Upon testing the pSG-ORF3(S80A) point mutant with pMT-ORF2 and immunoprecipitation with anti-ORF2 antibodies, only ORF2 was detected. All appropriate binding (Fig. 7, lanes 4–6 and 8) and expression (lanes 1–3) controls used in these experiments are shown. These results very clearly show that Ser80 is indispensable for the ORF2-ORF3 interaction.

Subsequently, we tested for ORF2 interaction with full-length ORF3, the 25-amino acid interaction domain of ORF3, and the S80A point mutation of ORF3 by an in vitro coupled transcription-translation immobilization assay. The full-length
ORF2 protein was cloned into the pRSET cloning vector, thus expressing a fusion protein with a His$_6$ tag fused to its N-terminal end. The ORF2 protein was immobilized to Ni$^{2+}$-NTA-charged beads for these experiments. The full-length ORF3 protein and the BD-ORF3-(57–81) (containing the 25-amino acid interaction domain) and ORF3(S80A) point mutation constructs were individually transcribed and translated using [$^{35}$S]methionine. Fig. 8 (A and B) shows the results from these experiments. Lanes 1 and 7 show the full-length ORF2-ORF3 interaction in vitro after radiolabeled ORF3 was allowed to bind to immobilized ORF2, washed, and analyzed by SDS-PAGE. Lane 4 shows the positive interaction of the full-length ORF2 protein with anti-ORF2 antibodies. COS-1 cells coexpressing ORF2 and ORF3 proteins are shown in lanes 10 and 7, respectively. The ORF3(S80A) mutant was not detected by immunoprecipitation of the ORF2 protein (lane 9). Lanes 1–6 and 8 are appropriate expression and binding controls for experiments analyzed on 8% (A) and 15% (B) SDS-polyacrylamide gels. Both anti-ORF2 and anti-ORF3 antibodies (Ab) were polyclonal antibodies. Lane M shows molecular mass markers (in kilodaltons) as indicated.

Ser$_{80}$ of ORF3 is conserved in all known isolates of HEV, except the Mexican isolate (33). We thus cloned the ORF3 coding region of the Mexican isolate of HEV into the yeast two-hybrid BD vector and tested it for an interaction against the AD-ORF2 (full-length) protein. The results for this experiment, we used the ORF3(57–81) protein (containing the 25-amino acid interaction domain) and the ORF3(S80A) point mutant, respectively. The Mexican ORF3 protein clearly showed negative upon yeast two-hybrid assays. Hence, we have proved through various in vivo and in vitro methods that Ser$_{80}$ is essential for the ORF2-ORF3 interaction.

Phosphorylation at Ser$_{80}$ of the ORF3 Protein Is Essential for the ORF2-ORF3 Interaction—Ser$_{80}$ of ORF3 has been shown to be the major site for phosphorylation (17). Our experiments described above showed that Ser$_{80}$ is essential for the ORF2-ORF3 interaction. We thus designed assays using λ-protein.
Fig. 8. A and B, in vitro coupled transcription-translation assays for detecting heterotypic interactions of the full-length HEV structural proteins ORF2 and ORF3 and their deletions. B represents Ni\(^{2+}\)-NTA beads in the figure. The His\(_{6}\)-ORF2 protein was produced by coupled transcription-translation in the absence of \(^{32}\)P(Met and immobilized on Ni\(^{2+}\)-NTA beads. Full-length ORF3, BD-ORF3(57–81), and ORF3(SS80A) were transcription-translated in the presence of \(^{35}\)S(Met and tested for interaction as shown in lanes 1, 7, and 8, respectively. Appropriate expression and binding controls are shown in lanes 2, 3, 5, and 6. C, a yeast two-hybrid comparison of interactions between the full-length ORF2 protein and ORF3(57–81), ORF3(SS80A), and the Mexican ORF3 isolate is shown. SDHis- and β-Gal assay represent the two-hybrid assays. The numbers represent liquid β-galactosidase assay results.

phosphatase to investigate the requirement of phosphorylation of Ser\(^{80}\) for the ORF2-ORF3 interaction. In these experiments, two aliquots of COS-1 cells were starved for phosphate and subjected to Ni\(^{2+}\)-NTA beads in the figure. The His\(_{6}\)-ORF2 protein was produced by coupled transcription-translation in the absence of \(^{32}\)P(Met and immobilized on Ni\(^{2+}\)-NTA beads. Full-length ORF3, BD-ORF3(57–81), and ORF3(SS80A) were transcription-translated in the presence of \(^{35}\)S(Met and tested for interaction as shown in lanes 1, 7, and 8, respectively. Appropriate expression and binding controls are shown in lanes 2, 3, 5, and 6. C, a yeast two-hybrid comparison of interactions between the full-length ORF2 protein and ORF3(57–81), ORF3(SS80A), and the Mexican ORF3 isolate is shown. SDHis- and β-Gal assay represent the two-hybrid assays. The numbers represent liquid β-galactosidase assay results.

Fig. 9. Phosphatase assays used to study the effects of phosphorylation on the ORF2-ORF3 interaction. Reactions with a plus sign represent λ-protein phosphatase treatment. \(^{32}\)P-Labeled ORF3 protein was immunoprecipitated using anti-ORF3 antibodies and allowed to interact with \(^{35}\)S-labeled ORF2 in vitro (lane 6). Lane 5 shows complete phosphatase activity of the enzyme. Lanes 1–4 show all required expression and immunoprecipitation controls for these experiments.

We subsequently used the ORF2 mutants ORF2(N137A,N310A), ORF2(N310A,N562A), and ORF2(Δ2–34) described in Table I. ORF2(N137A,N310A) and ORF2(N310A,N562A) are double point mutations (Asn-to-Ala) at positions 137, 310, and 562 in different combinations. ORF2(Δ2–34) is an N-terminal deletion of the putative signal sequence of the ORF2 protein, preventing its transport into the endoplasmic reticulum and thus rendering it non-glycosylated.

Fig. 10B (lane 1) shows transient coexpression in COS-1 cells of the full-length ORF2 protein along with the two forms of ORF2: non-glycosylated (ORF2) and glycosylated (gORF2). Lane 2 shows tunicamycin inhibition of glycosylation, with only the non-glycosylated form of ORF2. Lanes 4–6 correspond to expression of the non-glycosylated forms of ORF2 by mutants ORF2(N137A,N310A), ORF2(N310A,N562A), and ORF2(Δ2–34), respectively. All samples were co-immunoprecipitated using antibodies against the ORF2 protein. When these coexpression lysates were analyzed for the ORF3 protein, each one of the corresponding lysates containing the non-glycosylated form of ORF2 pulled out the ORF3 protein from the lysates (Fig. 10C). This clearly proves that the non-glycosylated form of ORF2 is capable of binding to ORF3. The combined results in this report prove that the phosphorylated ORF3 protein interacts preferentially with the non-glycosylated form of the ORF2 protein of HEV.

**DISCUSSION**

HEV cannot be cultured routinely, although it has recently been propagated in primary macaque hepatocytes (34, 35), and a virus resembling HEV has been cultured in A549 cells (33). As a result, studies of HEV protein synthesis, processing, and assembly have been limited to heterologous expression systems. We chose the yeast two-hybrid system to study the heterotypic interactions of the two proteins encoded by ORF2 and ORF3 located in the structural part of the HEV genome. Our interests in this interaction increased significantly when we found these proteins to colocalize upon immunofluorescence microscopy of cotransfected cells. Using the yeast two-hybrid approach, we showed interactions between these two proteins, mapped the interaction domains, and showed that Ser\(^{80}\) of ORF3 protein is responsible for this interaction. Results thus obtained were verified using other in vitro binding and immunoprecipitation techniques. Furthermore, we showed that the ORF2-ORF3 interaction is dependent upon phosphorylation at Ser\(^{80}\) of the ORF3 protein. Because the ORF2 protein exists in both glycosylated and non-glycosylated forms (25), we designed experiments to observe any preference shown by ORF3 for the phosphorylation of ORF3.
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We have shown that post-translational proteolytic cleavage is required for particle formation. Fraser et al. (36) have shown that the glycosylated ORF2 species is much more stable than non-glycosylated ORF2, which is present in the cytosol and represents the major product accumulated in the cytosol and represents the major product accumulated in the cytosol. It is postulated from this work that the non-glycosylated form may be involved in capsid assembly. Our results show that ORF3 preferentially binds to non-glycosylated ORF2, which is the one involved in capsid assembly. With ORF3 selectively binding to the non-glycosylated form of ORF2, which is the one involved in capsid formation, our study shows ORF3 to be an important candidate for participation in capsid assembly.

The stability of VLPs after proteolytic modification of the ORF2 protein has been shown to decrease (37). It has been thus postulated from previous work that recombinant HEV particles lack a stabilizing scaffold and thus become fragile and easily damaged during purification. This suggests the possibility of another protein interacting with ORF2 that may provide it more stability.

The ORF2 protein has been shown to self-associate in the absence of ORF3 to form dimers (12, 13) and VLPs (15, 37). Although these VLPs mimic the HEV virion, there are detectable differences in size, and the internal cavity thus formed is too small to accommodate the ~7.2-kb HEV genomic RNA (37). Virions of this size have not been found in the bile or stool of patients suffering from hepatitis E or of experimentally infected monkeys (1). Also, the calculated capacity of these VLPs for packaging RNA is only ~1 kb in size, whereas the size of the HEV genome is 7.5 kb. Together with the size assessment of the capsid and the calculated indispensable volume of the viral RNA, the possibility of the involvement of another viral protein, ORF3 in this case, may be a possibility for correct HEV capsid assembly. With ORF3 selectively binding to the non-glycosylated form of ORF2, which is the one involved in capsid formation, our study shows ORF3 to be an important candidate for participation in capsid assembly.

The ORF3 phosphoprotein has been shown to self-associate (13), to bind proteins containing SH3 domains, and to activate cellular MAPK (18). Although ORF3 maps in the structural region of the HEV genome, it has, in these recent reports, shown indications of regulatory functions. Also, with this report showing ORF2 interacting with ORF3, the possibility of a nonstructural function for the ORF2 protein also exists.

In this report, we have observed that the ORF3 protein from the Mexican isolate, which contains leucine instead of serine at position 80, fails to associate with ORF2. Upon close examination of the sequence of the Mexican ORF3 amino acid sequence in that region, we observed that it was considerably different, with an upstream serine at position 76 also replaced with leucine. Either the ORF2-ORF3 interaction is not critical for virion assembly or infection, as suggested by the Mexican HEV isolate, and has a nonstructural role, or the Mexican isolate has a weak serine-free ORF2-ORF3 interaction that we were unable to detect as fusion proteins in a yeast environment. In the absence of comparative infectivity data on the prototypic and Mexican isolates of HEV, it is difficult to assess the effects of lack of Ser⁴⁰ phosphorylation on viral pathogenesis. Finally, because the ORF2-ORF3 interaction is phosphorylation-
dependent, this seems like a good post-translational control mechanism to check the level of ORF2-ORF3 interaction within the hepatocyte.

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The Phosphorylated Form of the ORF3 Protein of Hepatitis E Virus Interacts with Its Non-glycosylated Form of the Major Capsid Protein, ORF2
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