Self-Association and Mapping of the Interaction Domain of Hepatitis E Virus ORF3 Protein

SHWETA TYAGI, SHAHID JAMEEL, AND SUNIL K. LAL*
Virology Group, International Centre for Genetic Engineering & Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

Received 18 September 2000/Accepted 30 November 2000

Hepatitis E virus (HEV) is a major human pathogen in the developing world. In the absence of an in vitro culture system, very little information on the basic biology of the virus exists. A small protein (~13.5 kDa) of unknown function, pORF3, is encoded by the third open reading frame of HEV. The N-terminal region of pORF3 is associated with the cytoskeleton using one of its hydrophobic domains. The C-terminal half of pORF3 is rich in proline residues and contains a putative src homology 3 (SH3) binding domain and a mitogen-activated protein kinase phosphorylation site. In this study, we demonstrate that pORF3 can homodimerize in vivo, using the yeast two-hybrid system. We have isolated a 43-amino-acid interaction domain of pORF3 which is capable of self-association in vivo and in vitro. The overlap of the dimerization domain with the SH3 binding and phosphorylation domains suggests that pORF3 may have a dimerization-dependent regulatory role to play in the signal transduction pathway.

Hepatitis E virus (HEV), the causative agent of hepatitis E, is a waterborne pathogen which is responsible for sporadic infections as well as large epidemics of acute viral hepatitis in developing countries (4, 5, 16, 17, 18, 25, 26, 35). The HEV genome organization resembles that of many alphaviruses, with nonstructural genes at the 5′ end and structural genes at the 3′ end (18, 27). It has a single-stranded positive-sense RNA genome of about 7.5 kb with three forward open reading frames (ORF1, ORF2, and ORF3) encoding three different proteins (19, 28, 31, 32). ORF1 (5,079 bp) is at the 5′ end of the genome and is predicted to code for a polyfunctional regulatory protein. ORF2 and ORF3 have been expressed in Escherichia coli, animal cells, baculovirus and yeast and in vitro in a coupled transcription-translation system (10, 12, 20, 24). ORF2 encodes the major HEV structural protein (pORF2), 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. When expressed through the baculoviral expression system, pORF2 was shown to assemble into virus-like particles which were cell associated as well as secreted in the culture medium (16, 22, 33).

ORF3 encodes a small, 13.5-kDa cytoskeleton-associated phosphoprotein (pORF3) (2, 36), which is expressed intracellularly and shows no major processing. The location of ORF3 on the 3′ end of the genome classifies it as a structural protein, though its function is yet to be defined.

In earlier expression studies, a dimeric form of pORF3 was always observed by using polyacrylamide gel electrophoresis (12). In its primary sequence, pORF3 contains two distinct hydrophobic domains in its N-terminal region, amino acids 16 to 32 and 37 to 62. These may support homodimerization of the protein. We have used the yeast two-hybrid system (7, 8) to show the homodimerization of pORF3. We have subsequently identified the domains of pORF3 that are involved in these homotypic interactions. Surprisingly, the homodimerization domain maps in a single stretch of 43 amino acids at the C-terminal end of pORF3. This region contains a smaller hydrophobic domain (amino acids 84 through 95) and two domains rich in proline residues. By constructing GAL4 activation and binding domain fusions of truncated ORF3 proteins, we have shown, using the in vivo yeast two-hybrid assay and in vitro immunoprecipitations, that this 43-amino-acid interaction domain of pORF3 is capable of self-association. The possible role of the homodimerization domain with respect to its position on the ORF3 protein has been postulated.

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 (6, 7, 11), and for mapping specific domains involved in protein-protein interactions (14, 21, 30). In this system, two plasmid-borne gene fusions are cotransformed into yeast cells and the interaction between two proteins is measured by the reconstitution of a functional transcriptional activator that triggers the expression of reporter genes lacZ and HIS3. We have used this system to study the self-association of pORF3 by cloning the DNA binding domain and the transcriptional activation domain upstream and in frame with the ORF3 sequence.

Homotypic interactions of pORF3. The full-length ORF3 of HEV was excised from the pSG-ORF3 vector (12) by a Smal and BamHI digestion and cloned into the yeast two-hybrid vectors (Table 1), resulting in an N-terminal in-frame fusion of either the GAL4 DNA binding domain or the GAL4 activation domain with the pORF3 ORF.
domain to ORF3. All constructs were verified by restriction digestion and sequencing. DNA manipulations were carried out as described by Sambrook et al. (29).

Saccharomyces cerevisiae Y190 (MATa trpl-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80URA3::GAL-lacZ LYS2::GAL-HIS3) cells were transformed with single plasmids or were cotransformed with the GAL4 BD and AD vectors containing ORF3. The Y190 host strain containing pAS2-SNF1 and pACT2-SNF4 was used as a positive control (9) and was kindly supplied by Stephen Elledge. Y190 contains integrated copies of both HIS3 and lacZ reporter genes under the control of GAL4 binding sites.

The results of the two-hybrid assay are shown in Fig. 1. Single transformants used in this assay were yeast (Y190) cells containing BD-ORF3, Y190 cells containing AD-ORF3, Y190 cells containing only the BD vectors, and Y190 cells containing only the AD vectors (Fig. 1A). All these transformants grew on the yeast extract-peptone-dextrose (YPD) plate (nonselective media) (Fig. 1B). Single transformants containing the BD vector by itself or as a fusion showed growth on the synthetic dextrose Trp \(^2\) (SD Trp \(^2\)) plate (Fig. 1C). Single transformants containing the AD vector by itself or as a fusion showed growth on the SD Leu \(^-\) plate (Fig. 1D). The cotransformants were similarly

---

**TABLE 1. Yeast strains, plasmids, and recombinant plasmid constructs used in this study**

<table>
<thead>
<tr>
<th>Strain, plasmid, or construct</th>
<th>Genotype or description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
</tr>
<tr>
<td>Y190</td>
<td>MATa trpl-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80URA3::GAL-lacZ LYS2::GAL-HIS3</td>
</tr>
<tr>
<td>PJ69-4a</td>
<td>MATa trpl-901 leu2-3,112 ura3-52 his3-200 gal4 gal80A Lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</td>
</tr>
<tr>
<td>PJ69-4o</td>
<td>MATa trpl-901 leu2-3,112 ura3-52 his3-200 gal4 gal80A Lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pGAD424</td>
<td>GAL4 AD vector [GAL4(788-881)]; LEU2, 2μm, Amp'</td>
</tr>
<tr>
<td>pGBT9</td>
<td>GAL4 DNA-BD vector [GAL4(1-147)]; TRP1, 2μm, Amp'</td>
</tr>
<tr>
<td><strong>Constructs</strong></td>
<td></td>
</tr>
<tr>
<td>pGBT9-ORF3</td>
<td>pSG-ORF3 digested with SmaI and BamHI, fragment ligated</td>
</tr>
<tr>
<td>pGAD424-ORF3</td>
<td>pSG-ORF3 digested with SmaI and BamHI, fragment ligated</td>
</tr>
<tr>
<td>pGBT9-ORF3\Delta56-123</td>
<td>pGBT9-ORF3 digested with BstII and BamHI and religated</td>
</tr>
<tr>
<td>pGAD424-ORF3\Delta80-123</td>
<td>pGAD-ORF3 digested with EagI and BamHI and religated</td>
</tr>
<tr>
<td>pGAD424-ORF3\Delta1-80</td>
<td>pGAD-ORF3 digested with EagI and XmaI and religated</td>
</tr>
<tr>
<td>pGAD424-ORF3\Delta1-80,100-123</td>
<td>pGAD1-80ORF3 digested with BsuUI and EcoRI and religated</td>
</tr>
<tr>
<td>pGBT9-ORF3\Delta1-80</td>
<td>pGBT-ORF3 digested with EagI and XmaI and religated</td>
</tr>
</tbody>
</table>

---

**FIG. 1. Two-hybrid results showing full-length homotypic interactions of the HEV ORF3 protein.** (A) Template for panels B through F, showing the clones streaked in each section of the plate. (B to E) Growth on YPD, SD Trp \(^-\), SD Leu \(^-\), and SD Trp \(^-\) Leu \(^-\) His \(^+\) medium plates, respectively. (F) Results from the β-galactosidase filter assay. Growth is represented by white on the black background of the petri dish. Blue is represented by the black streaks on a white background from nitrocellulose membrane.
plated on YPD and synthetic dextrose medium lacking Trp or Leu or lacking Trp, Leu, and His (SD Trp− Leu− His−), to select for clones in which the HIS3 gene was transactivated. Growth of the cotransformants in both SD Trp− and SD Leu− plates simply showed that both plasmids were present in the transformed cells. Growth of these clones on the SD Trp− Leu− His− media showed that the transcription of the HIS3 gene was turned on by the reconstitution of the GAL4 transactivator due to a specific protein-protein interaction (Fig. 1E). Colonies were transferred to nitrocellulose filters, and a β-galactosidase filter assay was performed as described earlier (3, 7) (Fig. 1F). The cotransformants containing both the BD-ORF3 and AD-ORF3 constructs along with the positive control used in this assay showed His prototrophy and β-galactosidase activity on the filter assay.

The liquid β-galactosidase assay was conducted and activity was determined using the substrate chlorophenol red-β-D-galactopyranoside as described previously (3, 23). The host strain Y190 alone, along with single transformants containing AD-ORF3 and BD-ORF3 and cotransformants containing AD and BD vectors without a fusion protein, BD vector and AD-ORF3, and BD-ORF3 and AD vectors, was used as a negative control showing almost no β-galactosidase activity. BD-SNF1/AD-SNF4 was the positive control, and the clones containing BD-ORF3 and AD-ORF3 were the test samples (Fig. 2). Relative enzymatic activity was determined in five independent transformants from each group. Our results from this assay indicate a strong protein-protein interaction between the AD-ORF3 and BD-ORF3 proteins.

The specificity of the pORF3-pORF3 interaction was confirmed using a yeast genetic assay for reconfirming positive two-hybrid interactions (34). Plasmid constructs were extracted from the positive Y190 cotransformants (BD-ORF3 and AD-ORF3, clone no. 1 and clone no. 2, shown in Fig. 1). The plasmids isolated from these clones were separated and verified using E. coli HB101 cells on M9 synthetic media lacking Leu. Subsequently, these plasmids were singly transformed into the PJ69-4a and PJ69-4α haploid yeast strains, kindly provided by Philip James (13). After genetic crossing, the His3 prototrophy of the diploid strains was tested by plating for

FIG. 2. Liquid β-galactosidase assay results. Single transformants and cotransformants were analyzed for a liquid β-galactosidase assay and were compared to each other. Values are given in arbitrary units. The numbers above each bar represent the mean of five independent transformants.

FIG. 3. Genetic verification of ORF3 homodimerization. The haploid host cell is designated per its mating type, α or α. Diploid cells are designated α/α. Growth of colonies is shown on YPD (nonselective) and SD Trp− Leu− His− media.
growth or blue color; - signifies no growth or blue color. His represents growth on SD Trp `Leu `His` media. The numbers in brackets show relative β-galactosidase units from the liquid β-galactosidase assay. AT signifies growth on SD Trp `Leu `His` media with 50 mM AT. Dip His represents growth of diploids tested through the genetic two-hybrid assay.

From the above experiments, it is clear that pORF3 forms a homodimer in vivo. As mentioned earlier, there are distinct hydrophobic domains present in ORF3 that may be responsible for homodimerization. Studies on the molecular dissection of pORF3 were thus continued to map the dimerization domain.

A 43-amino-acid region of the ORF3 protein is involved in dimerization. To characterize the homodimerization domain of the ORF3 protein, various deletions were designed, pGBT9-ORF3Δ56-123, pGAD424-ORF3Δ80-123, pGAD424-ORF3Δ1-80, pGAD424-ORF3Δ1-80,100-123, and pGAD424-ORF3Δ1-100 were constructed by restriction digestion and religation of the full-length constructs GBT-ORF3 and GAD-ORF3 as described in Table 1.

Our two-hybrid experiments consisted of cotransformation of one of the pORF3 truncated fusion proteins together with the corresponding fusion protein containing either full-length or truncated ORF3. The results of these experiments are shown in Fig. 4. BD-ORF3Δ56-123 and AD-ORF3Δ80-123, when cotransformed with their corresponding full-length ORF3 constructs (AD-ORF3 and BD-ORF3, respectively), showed no reporter gene activity. However, the ORF3Δ1-80 deletion, when cotransformed with its corresponding full-length ORF construct (BD-ORF3), showed strong reporter gene activation.

We further investigated the strength of interactions by measuring the HIS3 reporter gene for the full-length and truncated ORF3 proteins in the presence of 50 mM 3-aminotriazole (AT). Hundredfold serial dilutions of log-phase cultures of clones expressing BD-ORF3 and AD-ORF3 and deletions of AD-ORF3 and BD-ORF3 and appropriate negative controls were plated on SD His° with 50 mM AT. These results indicated the strength of the protein-protein interactions as a function of His prototrophy. Cells containing both fusions of full-length ORF3 proteins showed growth up to $10^{-4}$ serial dilutions on the SD His° 50 mM AT plate, confirming strong homodimerization. The deletion ORF3Δ1-80 also showed growth at a $10^{-4}$ dilution, whereas BD-ORF3Δ56-123, AD-ORF3Δ80-123, AD-ORF3Δ1-80,100-123, and ORF3Δ1-100 showed no growth on SD His° 50 mM AT media. Results of this assay are shown in Fig. 4, where + denotes growth of the clone being tested after a $10^{-4}$ dilution. This clearly showed that the interactions between full-length pORF3 molecules were strong and that AD-ORF3Δ1-80 (containing amino acids 81 through 123) was sufficient to show a protein-protein interaction (with its corresponding fusion full-length BD-ORF3) of reporter strength equal to that of full-length ORF3 molecules.

Constructs pGAD424-ORF3Δ1-80,100-123 and pGAD424-ORF3Δ1-100 (Table 1) divide the 43-amino-acid C-terminal region of ORF3Δ1-80 into two almost equal parts of about 20 amino acids each. When these two constructs were tested against their corresponding BD-ORF3 full-length fusion proteins for homodimerization, these two peptides were unable to show a positive phenotype on the two-hybrid screen (Fig. 4). This suggests that the 43-amino-acid region represents the intact homodimerization domain. This 43-amino-acid binding domain contains a hydrophobic domain (between amino acids 84 and 95) and also contains two polyproline regions (amino acids 75 to 86 and 104 to 113). The truncated proteins ORF3Δ1-100 and ORF3Δ1-80,100-123 divide the 43-amino-acid binding domain into two equal halves, each containing one of the polyproline regions while the hydrophobic domain stays intact in ORF3Δ1-80,100-123. This explains the liquid β-galactosidase values for a small stretch of 18 amino acids (ORF3 amino acids 81 to 99) fused to AD, which were significantly higher (0.40) than those for its counterpart AD-ORF3Δ1-100. Thus, we propose that the hydrophobic domain within amino acids 84 through 95 is involved in dimerization but is not enough to show a positive signal in the two-hybrid system.

It may be possible that the yeast two-hybrid system is unable to detect an interaction as weak as this or that the fusion of approximately 140 amino acids (of GAL4) to an 18-amino-acid ORF3 region becomes undetectable by the system, thus falling below the minimum threshold required to turn on the HIS3 gene.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>His</th>
<th>β-Gal</th>
<th>AT</th>
<th>Dip His</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-ORF3 / AD-ORF3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BD-ORF3Δ56-123 / AD-ORF3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BD-ORF3 / AD-ORF3Δ80-123</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BD-ORF3 / AD-ORF3Δ1-80</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BD-ORF3 / AD-ORF3Δ1-80,100-123</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BD-ORF3 / AD-ORF3Δ1-100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
FIG. 5. The 43-amino-acid domain of HEV ORF3 is capable of self-association in vivo and in vitro. (A to D) In vivo results using the yeast two-hybrid assay. Text in boldface describes the growth media and/or assay used. The numbers in brackets in panel B represent relative \( \beta \)-galactosidase units in a liquid assay. (E) In vitro results using a coupled transcription and translation assay system.

**Self-association of the homodimerization domain.** Based on the above observations, we constructed vectors expressing only the 43-amino-acid putative homodimerization domain as GAL4 AD and BD fusion proteins. These constructs, BD-ORF3\( \Delta 1-80 \) and AD-ORF3\( \Delta 1-80 \), are described in Table 1. Along with appropriate negative and positive controls, we tested the dimerization activity of these constructs and compared it to that for the full-length BD-ORF3 and AD-ORF3 positive interaction clones. Cotransformants were obtained in yeast Y190 strains containing the 43-amino-acid fusion constructs BD-ORF3\( \Delta 1-80 \) and AD-ORF3\( \Delta 1-80 \). Cotransformants and the full-length ORF3 cotransformants (described previously) were simultaneously tested for growth on YPD, SD Trp\( ^{-} \), SD Leu\( ^{-} \) and SD Trp\( ^{-} \) Leu\( ^{-} \) in order to confirm actively growing cells and the presence of the BD vector, the AD vector, and both BD and AD vectors, respectively.

Figure 5A through D shows the results of our two-hybrid comparisons of full-length ORF3 homodimerization versus a truncated ORF3 protein containing only the C-terminal 43-amino-acid (81 to 123) homodimerization domain. Figure 5A and B show that both full-length and deletion clones are able to turn on the yeast two-hybrid reporter genes efficiently. Figure 5A shows the growth of both full-length and truncated cotransformants on SD His\( ^{-} \) media. Figure 5B shows positive \( \beta \)-galactosidase activity on the filter and liquid assays for both the full-length and 43-amino-acid fusion constructs. The quantitative liquid \( \beta \)-galactosidase assay (results of which are shown in brackets) showed that the C-terminal 43-amino-acid region of ORF3 homodimerizes with an affinity almost equal to that of its wild-type full-length ORF3 counterpart. Figure 5C shows the strength of the interactions measured on SD His\( ^{-} \) 50 mM AT selective media, thus exhibiting the strength of the protein-protein interaction. Figure 5D indicates the ability of diploids to grow on selective media after a genetic cross of the singly transformed haploid strains. This assay was conducted as described previously (34).

We also tested the homodimerization potential of full-length pORF3 and the C-terminal 43 amino acids by means of an in vitro-coupled transcription-translation assay. The ORF3 full-length construct (pSGORF3, encoding 123 amino acids of pORF3) and the activation domain fusion ORF3\( \Delta 1-80 \) (pAD424-ORF3\( \Delta 1-80 \), encoding 156 amino acids of the fusion protein) were used in a coupled in vitro transcription-translation system (TNT coupled reticulocyte lysate system; Promega) as per the manufacturer’s instructions. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, both full-length ORF3 and the 43-amino-acid C-terminal ORF3 fusion proteins showed homodimerization under fully denaturing conditions (with \( \beta \)-mercaptoethanol) and partially denaturing conditions (without \( \beta \)-mercaptoethanol) as shown in Fig. 5E. We have thus proved beyond doubt that the interaction domain for homodimerization of HEV ORF3 lies within amino acids 81 to 123.

The minor protein pORF3, encoded by ORF3 within the structural region of the viral genome, is a 123-amino-acid protein of unknown function (31). pORF3 has been shown to localize to the cytoplasm and does not get posttranslationally modified (20, 36). There are two large hydrophobic domains in the N terminus of ORF3, and one of them has been shown to be responsible for association of pORF3 with the cytoskeleton. The hydrophobic domain which we find to be involved in homodimerization is a clearly distinct region away from these large hydrophobic domains and maps in the C-terminal region of ORF3.

pORF3 is also a phosphoprotein with a consensus phosphorylation sequence between amino acids 78 and 83. Also, two polyproline regions are present in the C-terminal region (amino acids 75 to 86 and 104 to 113) of pORF3. One of these has recently been shown to bind a number of cellular proteins containing src homology 3 (SH3) domains (S. Jameel, unpublished results). The dimerization domain that we have shown in this study is also present in this C-terminal region of pORF3 (amino acids 81 through 123). The SH3 binding domains, the phosphorylation signal, and the dimerization domains overlapping with each other suggest that self-association of pORF3 may have a regulatory function. It is known that the SH3 domains are found in many cellular proteins involved in signal transduction.
transduction pathways. Together with other interaction domains, these are used to make critical protein-protein contacts essential for signal transduction. The phosphorylation of pORF3 by mitogen-activated protein kinase also suggests a possible role for this viral protein in signal transduction. Its association with the cytoskeleton using the N-terminal end of pORF3, as well as a possible binding of SH3-containing targets to its C-terminal end, suggests that homodimerization may have a regulatory role in signal transduction by controlling the ability of pORF3 to interact with SH3 domain-containing proteins. The steric possible hindrance resulting from pORF3 dimerization may preclude pORF3-SH3 interactions and thus preclude the downstream functions of pORF3 in the signaling pathway.

We gratefully acknowledge the generous gifts by Stephen Elledge of the yeast two-hybrid vectors and strains and by Philip James of the PJ69-4a and -4b yeast strains.

This work was supported by internal funds from ICGB.

REFERENCES


