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The Full-Length and N-Terminal Deletion of ORF2 Protein of Hepatitis E Virus Can Dimerize

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Hepatitis E virus is a human RNA virus containing three open reading frames. Of these ORF2 encodes, the major capsid protein (pORF2), may possess regulatory functions, in addition to a structural one. In this study, we have shown using the yeast two-hybrid system and *in vitro* immobilization experiments that full-length pORF2 is capable of self-association, thus forming a homodimer. Using mutational analysis we have studied dimerization of various truncated versions of the ORF2 capsid protein using the yeast two-hybrid system and supported our findings with *in vitro* immobilization experiments. Deletions of pORF2 reveal a loss of the dimerization potential for all deletions except an N-terminal 127-amino-acid deletion. Our studies suggest that the dimerization property of pORF2 may not be amino-acid sequence-dependent but instead a complex formation of a specific tertiary structure that imparts pORF2 its property to self-associate. © 2001 Academic Press

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 (1–8). The HEV genome organization resembles many alphaviruses, with nonstructural genes at the 5′ end and structural genes at the 3′ end (5, 9). 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, pORF1, pORF2, and pORF3, respectively (10–13). 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 (14–16). In the absence of a reliable culture system for HEV, fundamental studies involving pORF1's role in HEV replication have not been undertaken. ORF2 and ORF3 have been expressed in *E. coli*, animal cells, baculovirus, yeast, and *in vitro* in a coupled transcription-translation system (17–21). ORF2, which encodes the major HEV structural protein (pORF2), 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 (22). When expressed through the baculoviral expression system, a 111-amino-acid N-terminal truncated ORF2 protein, was shown to assemble into virus-like particles which were cell-associated as well as secreted into the culture medium (23).

Anti-HEV antibodies raised against a recombinant HEV fusion protein confer immunity to HEV infection in cynomolgus monkeys (13), but little is known about the structure of the viral particle or the dimerization and multimerization of the capsid protein. Such information is essential for studies on antibody specificities and pose major hurdles in the development and clinical evaluation of effective vaccines.

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 (24–26) and for mapping specific domains involved in protein–protein interactions (21, 27–29). In this system, two plasmid-borne gene fusions are cotransformed into yeast cells and the interaction between these fusion proteins is measured by the reconstitution of a functional transcriptional activator that triggers the expression of reporter genes *lac*Z and HIS3. We have used this system to show homodimerization of pORF2 by cloning the DNA-binding domain and the transcriptional activation domain upstream and in-frame with the ORF2 sequence.

The ORF2 protein, being the major capsid protein of HEV, is expected to possess the potential to self-associate. Whether the self-association capacity of pORF2 is retained in a yeast nuclear environment was

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TABLE 1

Yeast Strains, Plasmids, and Recombinant Plasmid Constructs Used in This Study

Strain/Plasmid/Construct	Genotype/Description
Strains	
Y190	MAT**a** *trp1–901* his3 leu2–3, 112 ura3–52 ade2 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3
PJ69.4a	MAT**a** *trp 1–901* leu2–3, 112 ura 3–52 his 3–200 gal 4 Δ gal80Δ Lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ
PJ69.4α	MATα *trp 1–901* leu2–3, 112 ura 3–52 his 3–200 gal 4 Δ gal80Δ Lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ
Plasmids	
pACT2	*GAL4* AD vector [*GAL4(768–881)*]; *LEU2*, 2 μm, Amp^r
pAS2	GAL4 DNA-BD vector [*GAL4(1–147)*]; *TRP1*, 2 μm, Amp^r
Constructs	
pAS2-ORF2	pMT-ORF2 digested with *Nco*I and *Bam*HI, fragment ligated.
pACT-ORF2	pMT-ORF2 digested with *Nco*I and *Bam*HI, fragment ligated.
pAS2-ORF2Δ266–660	pAS2-ORF2 digested with *Eco*RI and *Bam*HI and religated.
pAS2-ORF2Δ1–226	pAS2-ORF2 digested with *Eco*RI and *Bam*HI, fragment religated.
pACT2-ORF2Δ509–660	pACT2-ORF2 digested with *Bss*HI and *Bam*HI and religated.
pACT2-ORF2Δ1–508	pACT2-ORF2 digested with *Bss*HI and *Nco*I and religated
pACT2-ORF2Δ1–127	pACT2-ORF2 digested with NcoI and SalI and religated.
pACT2-ORF2Δ128–660	pACT2-ORF2 digested with *Sal*I and *Bam*HI and religated.
pAS2-ORF2Δ1–127	pAS2-ORF2 digested with *Sal*I, fragment ligated.
pSG-ORF2Δ1–127	pACT2ORF2Δ1–127 digested with *Eco*RI and *Bam*HI, fragment ligated into pSGI vector (Stratagene)
pSG-ORF2	described in: Jameel, S. *et al.* 1996. *J. Virol.* **70:**207–216.
pRSET-ORF2	described in: Jameel, S. *et al.* 1996. *J. Virol.* **70:**207–216.

our concern since we were subsequently interested in being able to map the homodimerization domain of pORF2 in order to answer structural questions pertaining to capsid assembly protein. After establishing full-length pORF2 dimerization *in vivo*, using the yeast two-hybrid system, a mutational analysis of this protein was conducted to identify regions containing the homodimerization domain responsible in this homotypic interaction. We have found that the capability of pORF2 to dimerize is completely abolished when truncated pORF2 was assayed for dimerization with the exception of an N-terminal 127-amino-acid deletion which retained the affinity to self-associate. Our results suggest that the dimerization property of pORF2 lies between the amino acids 128–660 and may not be an amino-acid sequence-dependent interaction but instead, a complex formation of a specific tertiary structure that imparts it the ability to self-associate.

MATERIALS AND METHODS

Strains, media, and plasmid constructs. The full-length ORF2 of HEV was excised from the pMT-ORF2 vector (18) by a *Nco*I and *Bam*HI digestion and cloned into the yeast two-hybrid vectors resulting in an N-terminal in-frame fusion of either the GAL4 DNA-binding domain or the GAL4 activation domain to ORF2. All strains, plasmids, and plasmid constructs used in this study are described in Table 1. All constructs were verified by restriction digestion and sequencing. DNA manipulations were carried out as described by Sambrook *et al.* (30).

Yeast two-hybrid techniques. The yeast two-hybrid assay was performed using the Y190 yeast strain, which was transformed with the appropriate plasmids, using the lithium acetate procedure and grown on SD plates in the absence of Trp and Leu. Protein interaction analysis was performed on SD plates without Leu, Trp, and His (SDLeu$^-$ Trp$^-$ His$^-$). After 3 days at 30°C, individual colonies were streaked out and tested for liquid and filter β-galactosidase activity, 50 mM 3-amino-1,2,3-trizole (3AT) assay and the diploid His$^-$ assay described earlier (26). The filter β-galactosidase assay, a parameter directly reflecting the strength of protein interactions, was performed by streaking doubly transformed yeast colonies onto filter paper and allowed to grow for 2 days on selection medium. Yeast was permeabilized by freezing the yeast-impregnated filter in liquid nitrogen, and thawing at room temperature. The filter was placed on a second filter presoaked in a 0.1 M phosphate buffer (pH 7.0) containing 300 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 0.27% β-mercaptoethanol. Filters were left for 48 h to develop a blue color, this color change being the indication of a positive interaction.

The liquid β-galactosidase activity was determined using the substrate chlorophenol red-β-D-galactopyranoside (CPRG) as described previously (32, 33). Relative enzymatic activity was determined in five independent transformants. Data for quantitative assays were corrected for yeast cell numbers and are the mean ± S.E.M. of triplicate assays. Appropriate positive/negative controls and buffer blanks were used. The Y190 host strain containing pAS2-SNF1 and pACT2-SNF4 were used as positive controls (31) and were kindly supplied by Stephen Elledge.

In vitro transcription/translation assay. The full-length ORF2 protein (pRSET-ORF2, encoding 681 amino acids pORF2 with N-terminal His_6-Histidine) and radiolabeled [^{35}S]methionine full-length ORF2 protein (660 amino acids) were expressed in two separate reactions using a coupled *in vitro* transcription-translation system (TNT-coupled reticulocyte lysate system; Promega) as per manufacturer's instructions. The nonradioactive pORF2 was then bound to Ni-NTA beads (Amersham Pharmacia Biotech) and washed with PBS three times. Radio-labeled pORF2 was then added to the same tube and incubated for 4 h at 4°C with gentle shaking. The

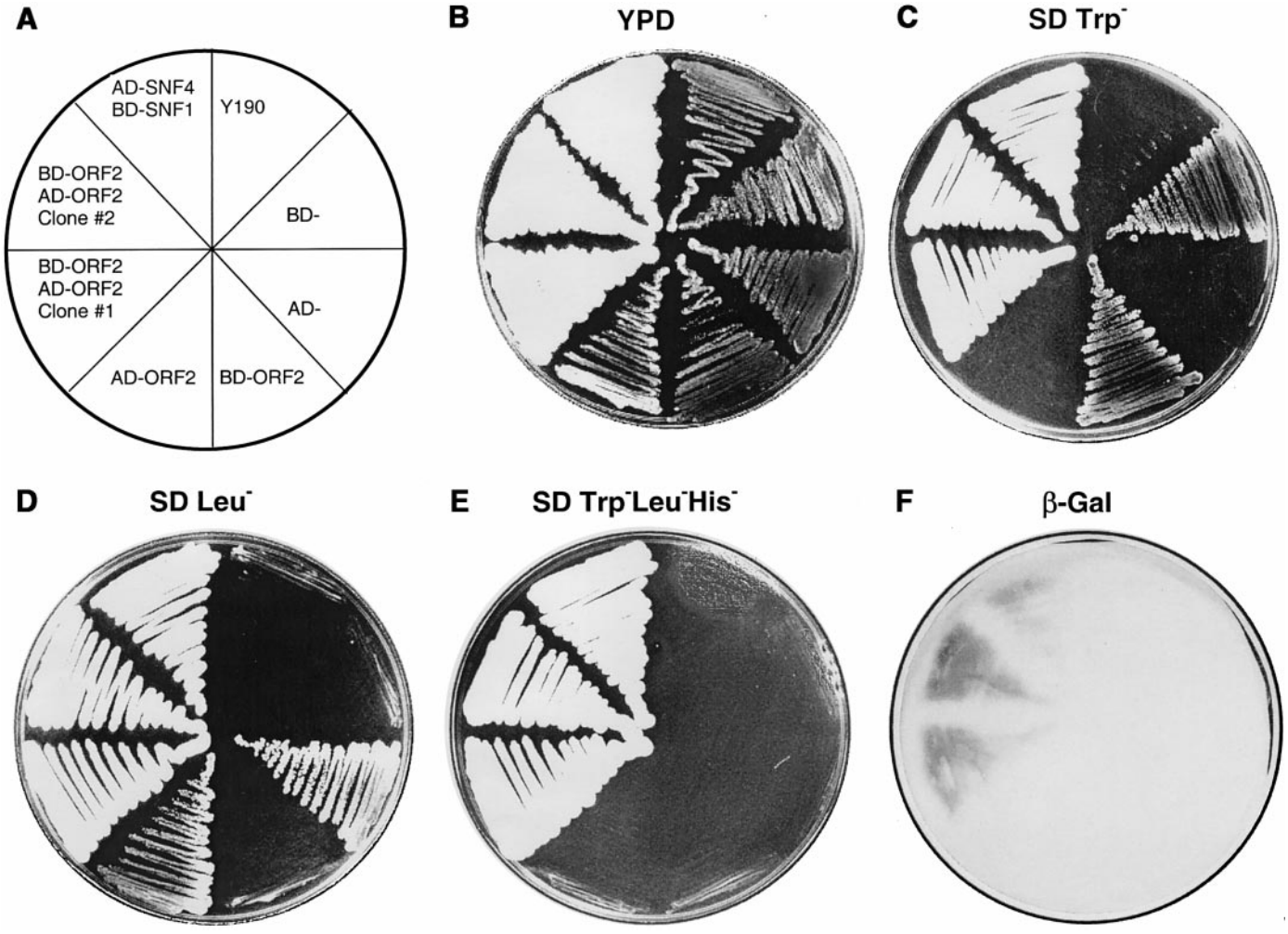

FIG. 1. Two-hybrid results showing full-length homotypic interactions of the HEV ORF2 protein. (A) A template for (B–F). (B, C, D, E, and F) Show growth on YPD, SDTrp$^-$, SDLeu$^-$, and SDTrp$^-$Leu$^-$His$^-$, and β-galactosidase filter assay, respectively.

beads were washed three times with PBS, resuspended in 10 μl of SDS–PAGE loading buffer and boiled for 5 min to dissociate the protein-bead complexes, and finally pelleted. Aliquots (10 μl) of the supernatants were subjected to SDS–PAGE, and the [^{35}S]methionine-labeled proteins were detected by autoradiography.

RESULTS

Homotypic interactions of pORF2. The full-length ORF2 of HEV was cloned into the yeast two-hybrid vectors (Table 1) resulting in an N-terminal in-frame fusion of either the GAL4 DNA-binding domain (GAL4 BD) or the GAL4 activation domain (GAL4 AD). *Saccharomyces cerevisiae* Y190 cells were transformed with single plasmids, or cotransformed with the GAL4 BD and GAL4 AD vectors containing ORF2. Y190 contains integrated copies of both HIS3 and *lac*Z reporter genes under the control of GAL4-binding sites. Single and cotransformants were plated on nonselective (YPD) and synthetic dextrose medium lacking tryptophan (SDTrp$^-$) or leucine (SDLeu$^-$) or tryptophan, leucine, and histidine (SD Trp$^-$Leu$^-$His$^-$) to select for clones in which the HIS3 gene was transactivated (Fig. 1). The Y190 host strain containing pAS2-SNF1 and pACT2-SNF4 was used as positive controls (31). Single transformants containing either the BD vector or the AD vector were able to grow on the SDTrp$^-$ and SDLeu$^-$ plates, respectively whereas only the cotransformants containing both plasmids and capable of activation of the HIS3 gene were able to grow on the SDHis$^-$ plates. Approximately 200 cotransformants were analysed for both His$^+$ and β-Gal$^+$ phenotypes. Two representative positive clones are shown in Fig. 1. These two clones were transferred to nitrocellulose filter for the β-galactosidase filter assay on which they turned blue.

Liquid β-galactosidase activity was determined for the positive clones along with all appropriate negative control clones using the substrate chlorophenol red-β-

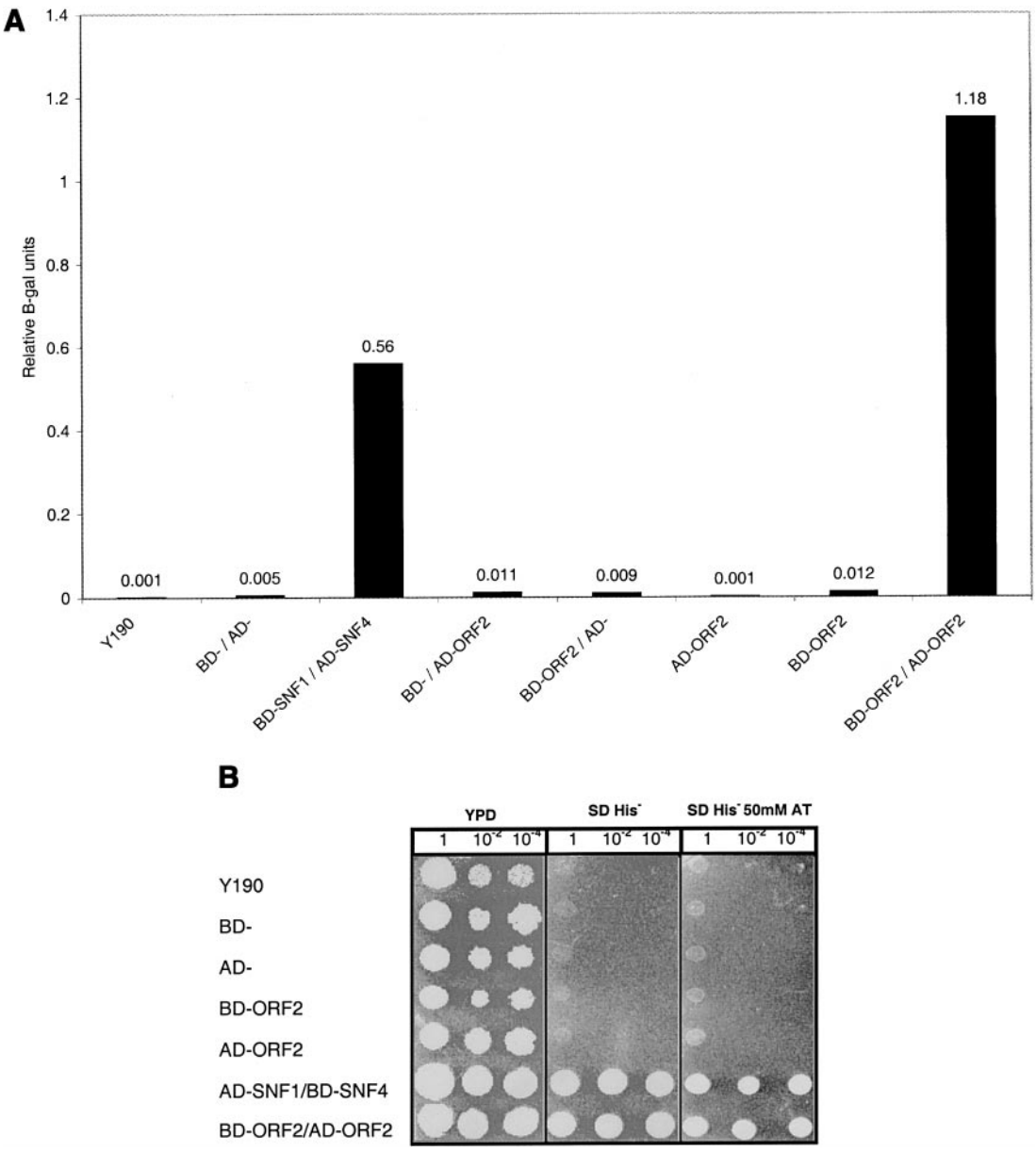

FIG. 2. (A) Liquid β-galactosidase assay results. Single and cotransformants were analyzed for a liquid β-galactosidase assay and were compared to each other. Values are given in arbitrary units. The numbers above each bar represent the mean of five independent transformants. (B) Measurement of the strength of pORF2–pORF2 interaction. Activation of the HIS3 reporter was determined for host (Y190) strain, single transformants (BD-, AD-, BD-ORF2, and AD-ORF2) and for cotransformants (AD-SNF1/BD-SNF4 and BD-ORF2/AD-ORF2). 100-fold serial dilutions of all the above mentioned log-phase cultures were plated on YPD (left panel), SDHis$^-$ (middle panel), and SDHis$^-$ with 50 mM AT (right panel).

D-galactopyranoside (CPRG) as described previously (32–34). Mean relative enzymatic activity was determined in five independent transformants and corrected for yeast cell number (Fig. 2A). These results very clearly show that the dimerization of pORF2 is a strong interaction.

We further investigated the level of activation of the HIS3 reporter genes for the full-length pORF2-pORF2 interaction in the presence of 50 mM AT on the SDHis$^-$ selection plates. Tenfold serial dilutions of log-phase cultures of Y190 strains expressing BD-SNF1 and AD-SNF4, BD-ORF2 and AD-ORF2 along with appropriate controls were plated on YPD, SDHis$^-$, and SDHis$^-$ with 50 mM 3AT (Fig. 2B). These results indicate the strength of the protein–protein interactions as a function of His prototrophy. The full-length pORF2–pORF2 interaction positive clone showed growth up to 10^{-4} serial dilutions on the His$^-$ 50 mM AT plate.

The specificity of the pORF2–pORF2 interaction was confirmed using a yeast genetic approach for recon-

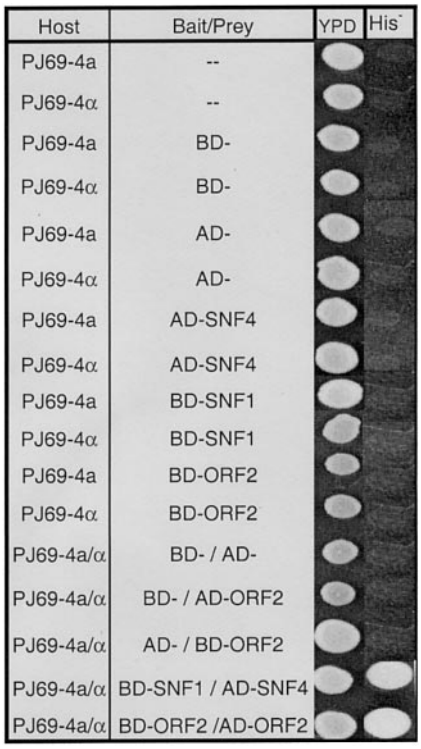

FIG. 3. Genetic verification of ORF2 homodimerization. Haploid host cell is designated as per its mating type: a or α. Diploid cells are designated a/α. Growth of colonies is shown on YPD and SDHis⁻ media.

firming positive two-hybrid interactions (26). Plasmid constructs were extracted from the positive Y190 co-transformants (BD-ORF2/AD-ORF2, Clone #1, and Clone #2). The plasmids isolated were separated and verified using *E. coli* HB101 cells on M9 synthetic media lacking leucine. Subsequently these plasmids were singly transformed into the PJ69-4a and PJ69-4α haploid yeast strains (35), kindly provided by Philip James. After genetic crossing, the His3 protrophy of the diploid strains were tested. Only the diploids containing both the BD-ORF2 and AD-ORF2 showed a positive phenotype similar to the positive-control clone BD-SNF1/AD-SNF4 diploid (Fig. 3).

From the above experiments it is clear that full-length pORF2 forms a homodimer *in vivo.* Studies on the molecular dissection of pORF2 were thus conducted to map regions within pORF2 responsible for the observed self-association.

A 127-amino-acid N-terminal deletion of pORF2 retains the ability to dimerize. To characterize the homodimerization domain of the ORF2 protein, various deletions were designed. pAS2-ORF2Δ226–660, pAS2-ORF2Δ1–226, pACT2-ORF2Δ509–660, pACT2-ORF2Δ1–509, pACT2-ORF2Δ1–127, pACT2-ORF2Δ128–660, and pAS2-ORF2Δ1–127 were constructed by restriction digestion and religation of the full-length constructs pAS2-ORF2 and pACT2-ORF2 as described in Table 1.

Our two-hybrid experiments consisted of cotransformation of one of the pORF2 truncated fusion-proteins together with the corresponding fusion protein containing full-length ORF2. The results of these experiments are shown in Fig. 4. The BD-ORF2Δ226–660, BD-ORF2Δ1–226, AD-ORF2Δ509–660, AD-ORF2Δ1–508, and AD-ORF2Δ128–660, when cotransformed with their corresponding full-length AD-ORF2 or BD-ORF2 construct, showed no reporter gene activity in all the yeast two-hybrid system experiments described for full-length pORF2–pORF2 interactions. However, the AD-ORF2Δ1–127 construct when cotransformed with its corresponding BD-ORF2 construct showed a positive for all our yeast two-hybrid experiments. Thus the amino acids 128–660 are required for the pORF2 to retain its dimerization potential.

Self-association of the homodimerization capable component of pORF2. Based on the above observations, we constructed vectors expressing only the 532-

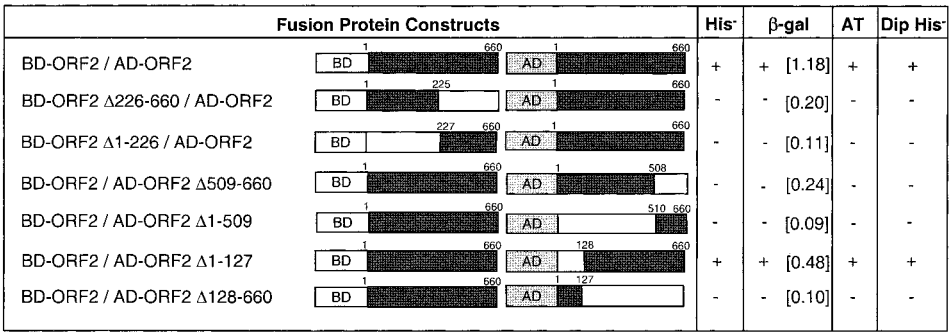

FIG. 4. Results of interactions of ORF2 deletion mutants with full-length ORF2, in the yeast two-hybrid system. Shaded boxes represent regions included in the respective constructs, whereas the open boxes represent regions that were deleted from the wild-type sequence. + signifies growth or blue color; − signifies no growth or blue color. His represents growth on SDTrp⁻Leu⁻His⁻ media. The numbers in brackets show relative β-galactosidase units from the liquid β-galactosidase assay. AT signifies growth on SDTrp⁻Leu⁻His⁻ media with 50 mM AT. Dip His represents growth of diploids tested through the genetic two-hybrid assay.

amino-acid (128–660) putative homodimerization domain as GAL4 AD and BD fusion proteins. These constructs, BD-ORF2Δ1–127 and AD-ORF2Δ1–127, 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 of full-length BD-ORF2 and AD-ORF2 interaction clones. Cotransformants were obtained in yeast Y190 strains containing the 532-amino-acid fusion constructs BD-ORF2Δ1–127 and AD-ORF2Δ1–127. Cotransformants and full-length ORF2 cotransformants (described previously) were simultaneously tested for growth on YPD, SDTrp$^-$, SDLeu$^-$, and SDTrp$^-$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.

Figures 5A through 5D shows the results of our two-hybrid comparisons of full-length ORF2 homodimerization versus the N-terminal 1–127 amino acid deleted ORF2 protein homodimerization. Figures 5A and 5B 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 SDHis$^-$ media. Figure 5B shows positive β-galactosidase activity on the filter and liquid assays for both the full-length and 127-amino-acid deleted pORF2 fusion constructs. The quantitative liquid β-galactosidase assay (results of which are shown in brackets) showed that the 127-amino-acid N-terminal truncated pORF2 homodimerizes with an affinity equal to its wild-type full-length ORF2 counterpart. Figure 5C shows the strength of the interactions measured on SDHis$^-$ 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 (13).

We also tested the homodimerization potential of full-length pORF2 and the N-terminal truncated pORF2 by means of an *in vitro* assay. The full-length pORF2 (pRSET-ORF2, encoding 660 amino acids pORF2 with N-terminal His_6-Histidine) and radiolabeled [^{35}S]methionine full-length pORF2 (pSG-ORF2, encoding 660 amino acids pORF2) were expressed in two separate reactions using the rabbit reticulocyte lysate coupled transcription-translation system (Promega) as per manufacturer's instructions. The nonradioactive His_6-pORF2 was then bound to Ni-NTA beads, washed with PBS three times, and equally aliquoted into two tubes. Radio-labeled full-length and the N-terminal 127-amino-acid deleted pORF2 were then added separately into these two tubes containing pORF2 immobilized on Ni-NTA beads. Appropriate control experiments were performed with tubes in which either the [^{35}S]methionine full-length pORF2 or the His_6-pORF2 was not added. After 4 h of incubation at 4°C with gentle shaking, the beads were washed three times with PBS, resuspended in 10 μl of SDS–PAGE loading buffer, boiled for 5 min to dissociate the protein-bead complexes, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The [^{35}S]methionine-labeled full-length ORF2 and the 127-amino-acid N-terminal deleted ORF2 protein were clearly visible by autoradiography (Fig. 5E) showing that they were capable of dimerizing with the immobilized pORF2 *in vitro*.

DISCUSSION

In this report we have shown that pORF2 of HEV is capable of self-association in a yeast cellular environment. Expression of pORF2 with an N-terminal truncation of 111 amino acids in the baculovirus system results in the production of virus-like particles (VLPs), which in contrast to synthetic peptides or full-length pORF2, appear to mimic the antigenicity and immunogenicity of the native virus (23, 34).

The ORF2 protein contains an N-terminal signal peptide that translocates it across the endoplasmic reticulum. This suggests the possibility that the N-terminal amino acids will not participate in the mature capsid protein. This observation holds true for both the baculovirus expressed as well as the N-terminal 127-amino-acid deletion of pORF2 that we have studied.

Upon careful quantitative analysis of the BD-ORF2/AD-ORF2Δ1–127 clone we find a liquid β-galactosidase activity of 0.48 units compared to that of full-length pORF2-pORF2 of 1.18 (Fig. 4). Although we have obtained a positive signal on the yeast two-hybrid system, we have decreased the interaction strength considerably. Also, as can be seen in Fig. 5, clones containing BD-ORF2Δ1–127/AD-ORF2Δ1–127 show a strong interaction (0.73 units) but it is lower than the full-length (1.18) liquid β-galactosidase readings. All this goes to prove that although we have a positive two-hybrid interaction with a 127-amino-acid deletion of ORF2, we are missing an essential portion of the protein which is probably between the amino acids 111–127 of the ORF2 protein.

Our data also suggests that larger deletions (greater than a 127-amino-acid deletion from the N-terminal of ORF2) result in a complete loss of the ability of this capsid protein to homodimerize. We hypothesize that dimerization of ORF2 is thus dependent on the secondary and tertiary structures formed by the three-dimensional folding of the protein rather than a sequence specific recognition of an interacting domain.

Another interesting observation from immunological studies reveals that the antibody reactivity and immunogenicity of VLPs have been attributed to conformational epitopes which are not present by synthetic peptides, full-length pORF2, and most HEV proteins

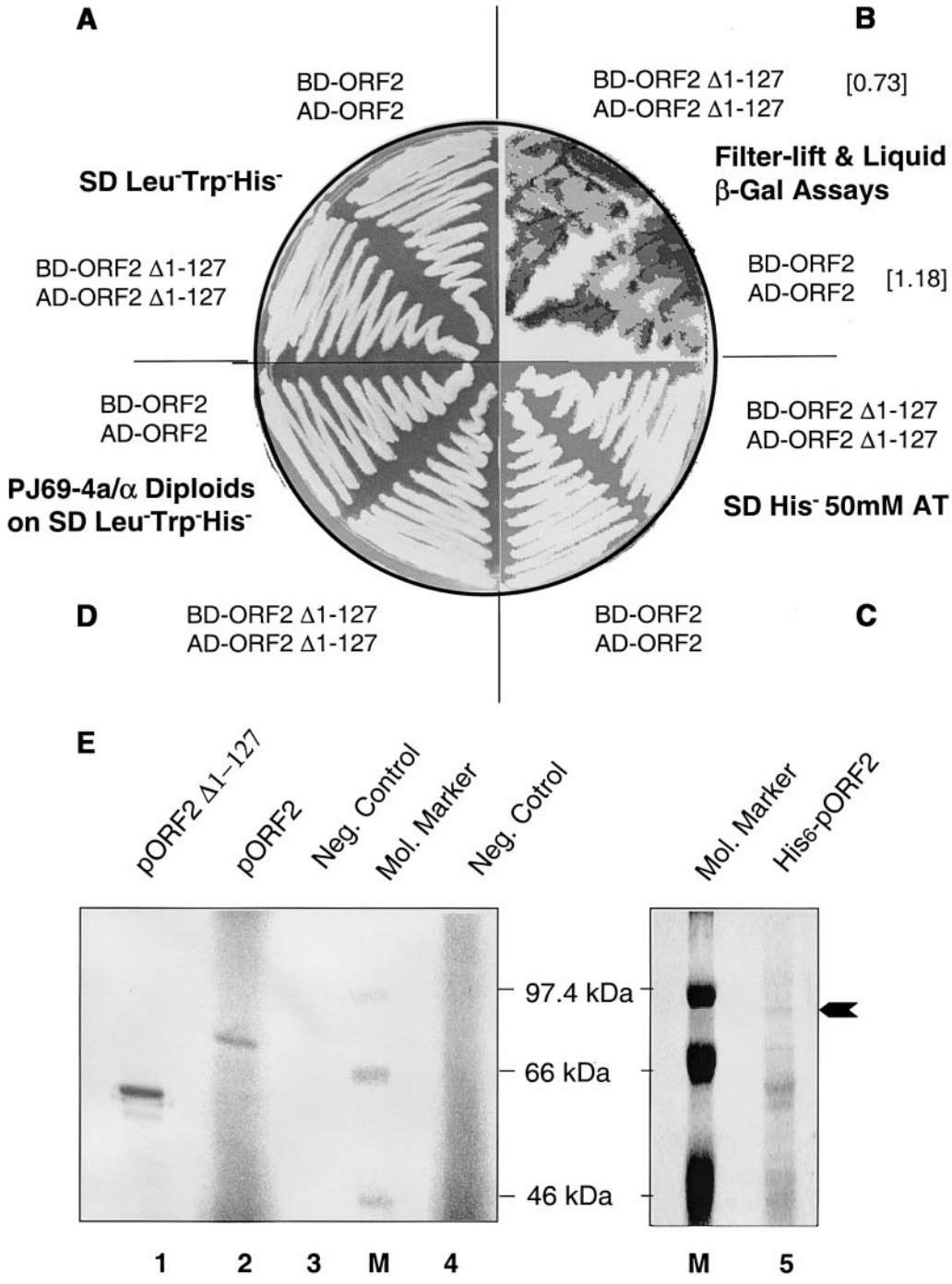

FIG. 5. The 532-amino-acid domain of HEV ORF2 is capable of self-association *in vivo* and *in vitro*. (A to D) *In vivo* results using the yeast two-hybrid assay. Text in boldface describes the growth media and/or assay used. The numbers in brackets in (B) represent relative *β*-galactosidase units in a liquid assay. (E) *In vitro* results using the coupled transcription and translation ORF2 immobilization assay. Lanes 1 and 2 show [^{35}S] radiolabeled Δ1–127pORF2 and full-length pORF2 eluted from beads to which nonradiolabelled His_6-ORF2 had been immobilized, respectively. Lanes 3 and 4 show results using [^{35}S] radiolabelled Δ1–127pORF2 or full-length pORF2, were separately added to the beads in the absence of His_6-ORF2 in the reaction tubes, beads washed, protein eluted, and electrophoresed, respectively. Lane 5 shows full-length His_6-ORF2 protein (◀) eluted from beads and stained with Commassie blue.

expressed in *Escherichia coli* (23, 34). This again goes in support of our findings that the pORF2 forms a complex secondary and tertiary structure critical for its immunological and self-association property. This complex three-dimensional structure gets disrupted in our deletion experiments (except ORF2Δ1–127) and thus renders the truncated pORF2 incapable of self-association.

It may be recalled that virus only use full-length capsid protein for headfull packaging resulting in a new virus particle. We feel that the loss of dimerization potential for a truncated pORF2 might be a mechanism for the virus to select for full-length pORF2 for self-assembly during capsid formation thus eliminating the use of truncated pORF2 resulting in defective viral capsids.

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