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Two Independent Pathways of Expression Lead to Self-Assembly of the Rabbit Hemorrhagic Disease Virus Capsid Protein

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The rabbit hemorrhagic disease virus capsid protein was expressed in insect cells either as an individual protein species, from a mRNA analogous to the viral subgenomic RNA, or as part of a polyprotein that included the viral 3C-like protease and the RNA polymerase. Both pathways of expression led to the assembly of viruslike particles morphologically and antigenically similar to purified virus.

The lethal rabbit hemorrhagic disease is caused by a recently characterized calicivirus (7, 18, 21) and affects mainly adult animals. The virions have a diameter of about 35 nm and a characteristic morphology due to a regular array of projections 5 to 6 nm long on the capsid surface. The rabbit hemorrhagic disease virus (RHDV) genome is a single-stranded, positive-polarity RNA molecule 7,437 nucleotides (nt) long. Its sequencing (15, 22) has revealed the presence of two open reading frames (ORFs). ORF1 (7,032 nt) encodes a polyprotein of 257 kDa that includes the capsid protein VP60 at its C terminus. This organization is the same as that of the european brown hare syndrome virus, which is closely related to RHDV (9, 24), but differs from that of other caliciviruses, in which the capsid protein gene is encoded by a separate ORF (8, 11, 13, 17). ORF2 is located at the 3′ end of the genome and encodes a putative protein of 117 amino acids, homologous to the ORF3 product in other caliciviruses (15). Besides the genomic RNA, an abundant subgenomic RNA of 2.2 kb that contains the entire VP60 gene and the ORF2 sequence is present. Both the genomic and subgenomic RNAs are polyadenylated and covalently linked to a 15-kDa protein (VPg) (16). It has been suggested that the subgenomic RNA is responsible for the synthesis of most or all of the VP60 assembled into mature virions (20). Whether proteolytic processing of the polyprotein synthesized from the genomic RNA yields VP60 protein competent for capsid assembly has remained a matter of speculation (4, 15, 20).

We addressed this issue by expression of VP60 via different baculovirus vectors. The system is suitable for the study of capsid assembly and may be used to produce an improved RHDV vaccine.

Experimental design. Figure 1 outlines two strategies used to achieve expression of VP60 in insect cells. Plasmid bVL-VP60 contains an insert corresponding exactly to the sequence of the subgenomic RNA under control of the polyhedrin promoter in pVL-1392 (Stratagene). It was constructed by subsequent ligations of a 93-bp PCR product starting with the sequence of the forward primer (GTTATGGAGGGCAAAG CCCG [underlined is the start codon of VP60, at positions 5305 to 5307 of the RHDV sequence]) and a *Bam*HI cDNA fragment, from nucleotide 5395 to the poly(A) tract of the RHDV sequence (4, 23). On the other hand, bVL-672 (4) and its derivatives (bVL-M4 and bVL-M1) were created by ligating into pVL-1393 (Stratagene) an *Eco*RI cDNA fragment (from nt 3077 to 7044 of RHDV). This insert encodes a polyprotein comprising the RHDV protease, polymerase, and capsid protein. Thus, to obtain VP60, the 3C-like protease must be able to cut the polyprotein with high specificity in the cytoplasmic milieu of insect cells, similarly to what observed in vitro and in *Escherichia coli* (4). In principle, these two pathways recapitulate the events occurring in RHDV-infected cells, where both the genomic and subgenomic RNAs are translated. The protease domain of bVL-M4 contains a cysteine-to-serine mutation that has been shown to reduce the in vitro activity of the protease to about 50% compared with the wild-type enzyme; bVL-M1 contains a cysteine-to-glycine mutation that completely abolishes the proteolytic activity (4).

Isolation of recombinant baculovirus expressing RHDV capsid protein. Several recombinant baculovirus clones were obtained for each transfer vector in accordance with standard procedures (19). One of each kind was chosen after testing for expression of the recombinant protein by enzyme-linked immunosorbent assay (ELISA) with anti-VP60 polyclonal rabbit serum (6, 7) and verification through DNA sequencing (Sequenase kit; Amersham) and Southern blotting (23).

The amount of VP60 in Sf9 cells infected at a multiplicity of infection of 10 with bVL-VP60 reached a maximum at the last day checked (day 5 postinfection) both in the culture medium and in cellular lysates (Fig. 2). That the 60-kDa protein corresponds to VP60 was confirmed by Western blot and by immunoprecipitation with anti-VP60 antibodies (data not shown). The relative amounts of VP60 in cellular extracts and in the culture medium indicate that it was easily released in the latter, reaching a concentration of about 100 μg/ml in 400-ml spinner cultures. Expression of recombinant products by the baculovirus clones containing the longer inserts (bVL-672, bVL-M4, and bVL-M1) was 10- to 20-fold lower than that of bVL-VP60 (data not shown), possibly because of the lower steady-state level of the longer mRNA and of the polyprotein or toxicity of the protease domain.

Synthesis of recombinant VP60 by two different pathways. The 60-kDa protein expressed in bVL-VP60-infected cells was recognized by anti-VP60 monoclonal antibody (MAb) 5G3 (5, 6, 24) and comigrated with capsid protein from purified virus (Fig. 3A). A larger, less abundant protein was also reproducibly detected (Fig. 3A, lane 2, and 3B, lane 1) after infection with bVL-VP60 but not after infection with the other vectors.

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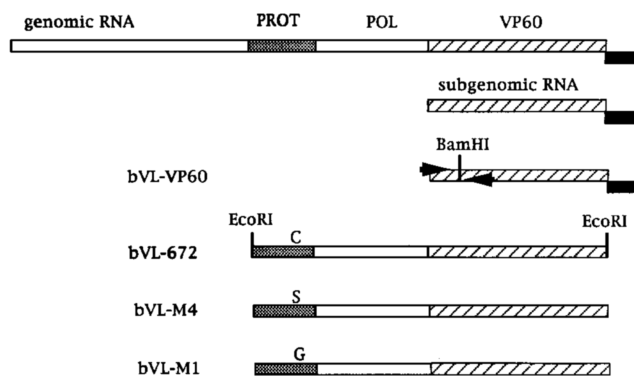

FIG. 1. Expression constructs. The 7,437-nt-long, polyadenylated RHDV genome is represented by the bar at the top. The positions of the genes for the 3C-like protease (dotted area), the RNA polymerase (empty box), and the capsid protein (striped box) and of ORF2 (black box) are indicated. The RHDV subgenomic RNA (2,143 nt) encodes the capsid protein gene and ORF2. The insert in bVL-VP60 corresponds to the subgenomic RNA and includes a poly(A) stretch. Its 5′ end was created by PCR amplification (opposite arrows) and digestion with *Bam*HI. The 3,967-bp insert in bVL-672, bVL-M4, and bVL-M1 contains the protease, polymerase, and capsid protein genes but not the ORF2 sequence. The protease domain of bVL-M4 and bVL-M1 differs from the wild-type sequence of bVL-672 by the introduction of single mutations, at amino acid 1212 of the RHDV sequence, as indicated (C, cysteine; S, serine; G, glycine). The viral ORF1 termination codon at the end of VP60 (nt 7042 to 7044) was present in all of the constructs.

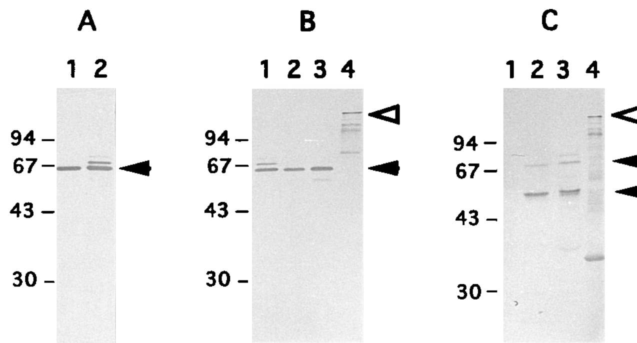

FIG. 3. Western blot analysis of recombinant products expressed in infected Sf9 cells. (A) Purified virus from infected rabbits (lane 1) and proteins in the medium of bVL-VP60-infected cells (lane 2) reacted with anti-VP60 MAb 5G3. The arrow indicates the major recombinant protein comigrating with RHDV VP60. (B) Extracts from Sf9 cells infected with bVL-VP60 (lane 1), bVL-672 (lane 2), bVL-M4 (lane 3), and bVL-M1 (lane 4) reacted with MAb 5G3. The black arrow indicates the position of the mature VP60 protein, and the white arrow indicates the position of the bVL-M1 uncut polyprotein precursor. Panel C is the same as panel B, except that the filter was reacted with C22 anti-RNA polymerase rabbit polyclonal serum. The black arrows point to the positions of the putative products of polyprotein processing (lanes 2 and 3) that contain the polymerase domain, and the white arrow shows the position of the bVL-M1 uncut polyprotein precursor. The relative migration of silver-stained molecular size markers (in kilodaltons) is reported to the left of each panel.

The nature of this protein remains to be determined, but it could not originate from translation starting at the mutated polyhedrin initiation codon (2) because this codon is not in frame with the VP60 ORF. Detection of VP60 from cells infected with bVL-672 and bVL-M4 (Fig. 3B, lanes 2 and 3) indicated that correct processing of the polyprotein by the protease domain was taking place. In bVL-M4, the protease activity was indistinguishable from that of the wild-type construct, although the Cys-to-Ser mutation crippled the protease in vitro and in *E. coli* (4). The possibility that the 60-kDa band did not originate through a specific proteolytic cut of the polyprotein was ruled out by detection of a band of the expected size (142 kDa) after expression of bVL-M1, which contains a null mutation of the RHDV protease (Fig. 3B, lane 4). The presence of several additional bands in bVL-M1 cell extracts suggests that the polyprotein is much less stable than the mature viral proteins. In bVL-672- and bVL-M4-infected cells, the presence of mature VP60 should be accompanied by equimolar amounts of the other proteolytic product(s). As expected, an anti-RHDV RNA polymerase serum (C22, raised against bacterially expressed polymerase), yielded a specific signal with extracts from bVL-672- and bVL-M4-infected cells but not with extracts from bVL-VP60-infected cells (Fig. 3C). Again, the intact polyprotein was detected in the bVL-M1 sample (Fig. 3C, lane 4). No signal at all was obtained in a control experiment performed with preimmune serum. Of the two proteins of about 55 and 73 kDa detected in the bVL-672 and bVL-M4 samples (Fig. 3C, lanes 2 and 3), the former is consistent with the putative size of the mature viral polymerase while the latter may correspond to a protease-polymerase hybrid protein, in analogy to the 3CD protein of picornaviruses (1, 12).

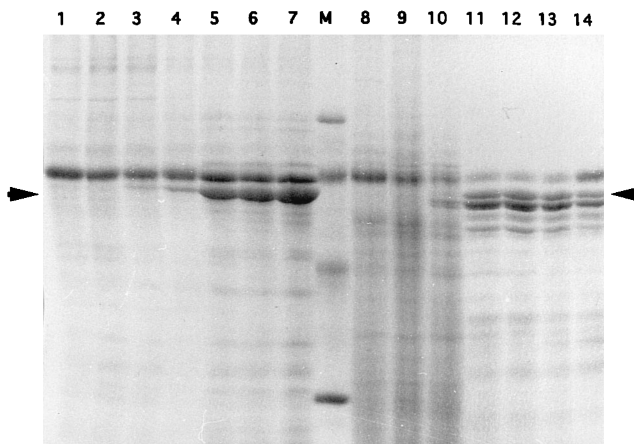

FIG. 2. Time course of expression of the recombinant VP60 protein in Sf9 cells infected with the bVL-VP60 vector (multiplicity of infection, 10). Equal amounts of total protein (10 μg) from culture medium (lanes 1 through 7) or from cellular lysates (lanes 8 through 14) were separated on a denaturing 10% polyacrylamide gel and stained with Coomassie blue. The proteins in the culture medium were subjected to polyethylene glycol precipitation prior to being loading onto the gel. Lanes: 1 to 7, proteins in the medium of mock-infected (lane 1) or bVL-VP60-infected cells at 0, 1, 2, 3, 4, and 5 days postinfection, respectively; 8 through 14, proteins in cellular extracts from mock-infected cells (lane 8) or bVL-VP60-infected cells (same order as above); M, molecular size markers (from the top, 94, 67, 43, and 30 kDa). The arrows point to the position of the recombinant VP60 protein.

Assembly of viruslike particles (VLPs). The assembly of recombinant VP60 was tested by analysis of sedimentation velocity (38,000 rpm for 100 min at 4°C in a Kontron TST41 rotor) in sucrose gradients (10 to 30% [wt/vol]). By monitoring fractions from each gradient by UV adsorption, ELISA, and Western blotting with the 5G3 anti-VP60 antibody (data not shown), we identified a major peak, with a calculated sedimentation coefficient of 118S, in bVL-VP60, bVL-672, and bVL-M4 samples but not in bVL-M1 samples. The same sedimentation coefficient was displayed by empty virions from a sample of RHDV run in parallel on an identical gradient; no recombinant material sedimented to the position occupied by complete RHDV particles (153S). Gel electrophoresis of the 153S (infectious virus) and 118S (recombinant material) fractions demonstrated that VP60 represented 95% of the total protein in these fractions (data not shown). VLPs were ob-

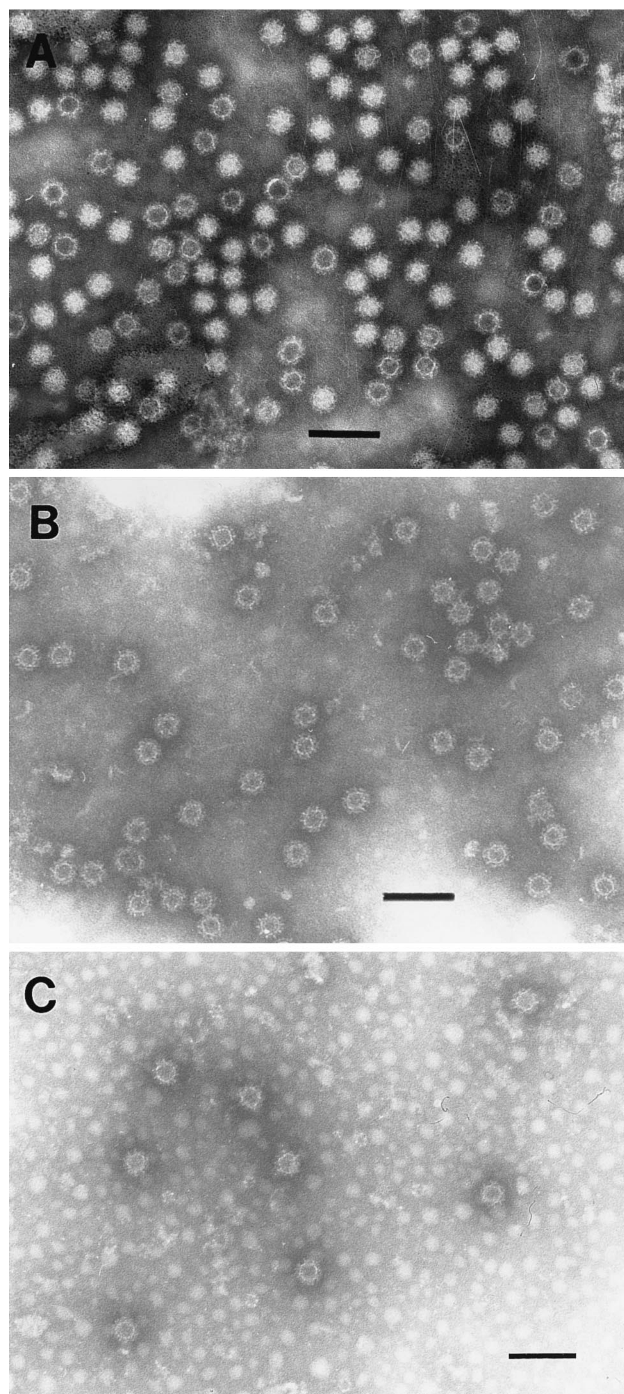

FIG. 4. Electron micrographs of negatively stained samples of purified RHDV showing both full electron-dense capsids and empty capsids (A), recombinant VLPs purified from bVL-VP60-infected cells (B), and recombinant VLPs from bVL-672-infected cells (C). Magnification, ×52,000; bar, 100 nm.

served by electron microscopy in the 118S fractions from bVL-VP60, bVL-672, and bVL-M4 but not in those from bVL-M1 (Fig. 4 and data not shown), proving that VP60 alone is sufficient to yield VLPs. Our results obtained with the bVL-VP60 construct are similar to those recently reported by others for RHDV (14) and Norwalk virus (10); in addition, we demonstrate here that VLP assembly occurs irrespectively of the

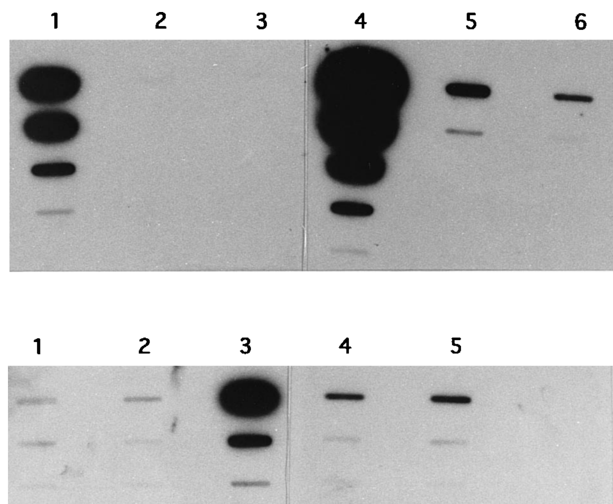

FIG. 5. Slot blot hybridization of an RHDV-specific cDNA probe to serial 10-fold dilutions of RNA from different sources. Columns 1 and 2: total RNA extracted from 1.3×10^7 purified RHDV particles and 6.5×10^7 purified bVL-VP60 recombinant VLPs, respectively. Columns 3, 5 and 6: poly $(A)^+$ RNA (starting at 100 ng) purified from mock-infected Sf9 cells and from Sf9 cells infected with bVL-VP60 or bVL-672, respectively. In column 4, serial 10-fold dilutions of plasmid DNA (p672) starting at 100 ng (equivalent to 1.1×10^7 molecules) were included as a control. Lower panel: slot blot hybridization to a total cDNA probe from uninfected Sf9 cells. The columns (from 1 to 5) represent the same samples as in the upper panel (with the exception of the plasmid DNA control) in the identical order.

pathway followed for the synthesis of VP60. As a consequence, RHD virions may well be constituted by a mixture of two VP60 species differing by two amino acids at the amino terminus (3) and originating from both the subgenomic and genomic RNAs.

Viruslike particles do not package RNA. The morphology of the VLPs was identical to that of RHDV but for the absence of an electron-dense core, suggesting that they did not contain nucleic acid. By slot blot hybridization (23) with an RHDV-specific probe, we estimated that fewer than 1 in 5,000 VLPs may package RNA containing the RHDV subgenomic sequence (Fig. 5). Furthermore, the VLPs do not appear to package cellular RNA as demonstrated by the lack of any signal above the background (compare columns 1 and 2) after hybridization to labelled cDNA from Sf9 cells. These data are consistent with the idea that VP60 per se does not have affinity for RNA. The failure to package transcripts originating from bVL-VP60 is not surprising; although the subgenomic RNA is efficiently packaged by RHDV in vivo (16), the 5′ ends of the two molecules differ by the addition of extra nucleotides from the polyhedrin gene untranslated region in the former and by the presence of a covalently linked viral protein (VPg) in the latter (16).

Antigenicity and immunogenicity of RHDV VLPs. ELISAs were performed as previously described (5), with equal amounts of antigens and a panel of anti-RHDV MAbs. The MAbs belong to two groups. MAbs 1H8, 3H2, 3H6, 6F9, and 6H6 recognize external epitopes, important for immunogenicity, while MAbs 6D6, 6G2, and 5G3 are directed towards internal epitopes (5). All of the viral epitopes (Fig. 6B) were preserved on the VLPs from bVL-VP60 (Fig. 6A) or bVL-672 (Fig. 6C). MAbs specific for internal epitopes displayed higher reactivity against the VLPs than to the virus, indicating the presence of some incompletely assembled VLPs. This was confirmed by their detection in electron micrographs, where they appear as open circles. The top fractions of the sucrose gradi-

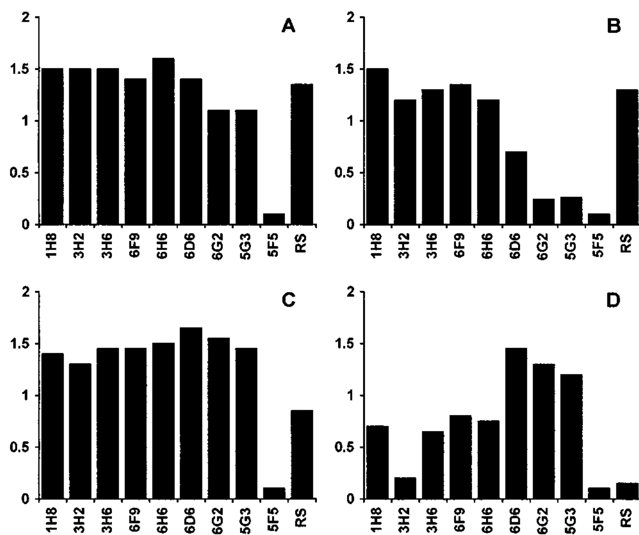

FIG. 6. Antigenicity of recombinant VLPs determined by sandwich ELISA. Sucrose gradient-purified VLPs from bVL-VP60 (A), purified RHDV (B), VLPs from bVL-672 (C), and the supernatant after pelleting of bVL-VP60 VLPs (D) were adsorbed onto microplates coated with anti-RHDV polyclonal serum and probed with eight anti-RHDV MAbs and with anti-RHDV hyperimmune rabbit serum (RS). Anti-foot-and-mouth disease virus MAb 5F5 was used as a background control. The values on the ordinates are A_{492} units.

ent used to purify the VLPs also contained VP60; the antigenic profile of this material (Fig. 6D) was identical to that of the putative RHDV subunits obtained from RHDV-infected livers (5), suggesting that the assembly process in insect cells is also faithful in this respect.

Immunization of six rabbits never exposed to RHDV was carried out with two doses (10 and 100 μg) of the bVL-VP60 recombinant VLPs. After a single intramuscular injection with aluminum hydroxyde as an adjuvant, within 2 weeks, all of the animals developed anti-RHDV titers in serum ranging from 1/60 to 1/400. After challenge with 10^3 50% lethal doses of infectious RHDV, two control rabbits died within 52 h while all of the immunized animals survived and remained asymptomatic. The effectiveness of the VLPs in the presence of a mild adjuvant opens interesting prospects for their use as a recombinant vaccine.

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