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Synthesis and Characterization of Chimeric Particles between Epizootic Hemorrhagic Disease Virus and Bluetongue Virus: Functional Domains Are Conserved on the VP3 Protein

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A functional assay has been developed to determine the conservative nature of the interacting sites of various structural proteins of orbiviruses by using baculovirus expression vectors. For this investigation, proteins of two serologically related orbiviruses, bluetongue virus (BTV) and the less studied epizootic hemorrhagic disease virus (EHDV), were used to synthesize chimeric particles. The results demonstrate that the inner capsid protein VP3 of EHDV-1 can replace VP3 protein of BTV in formation of the single-shelled corelike particles and the double-shelled viruslike particles. Moreover, we have demonstrated that all three minor core proteins (VP1, VP4, and VP6) can be incorporated into the homologous and chimeric corelike and viruslike particles, indicating that the functional epitopes of the VP3 protein are conserved for the morphological events of the virus. This is the first evidence of assembly of seven structural proteins of the virus by a baculovirus expression system. Confirmation at the molecular level was obtained by determining the EHDV-1 L3 gene nucleic sequence and by comparing it with sequences available for BTV. The analysis revealed a high degree homology between the two proteins: 20% difference, 50% of which is conservative. The consequences for *Orbivirus* phylogeny and the possibility of gene reassortments are discussed.

Epizootic hemorrhagic disease virus (EHDV) is an arbovirus of the *Reoviridae* family, *Orbivirus* genus, and is related to bluetongue virus (BTV), the *Orbivirus* prototype. Bluetongue is a disease of sheep, while EHDV mainly causes a hemorrhagic disease in white-tailed deer. EHDV has been isolated in many parts of the world (e.g., the United States, Canada, Japan, Australia, South Africa, and Nigeria). In the United States, the presence of both BTV and EHDV is of concern to the animal industries as it imposes restrictions on the international movement of livestock. Also, the economic losses attributable to the often subacute disease are difficult to estimate. Both viruses are transmitted principally by *Culicoides* vectors to the vertebrate ruminant hosts. Similar clinical signs of disease are observed in cattle infected with either BTV or EHDV. Moreover, EHDV has also been isolated recently from clinically normal, naturally infected sheep. Therefore, the extent of EHDV infection in domestic ruminants has probably not been estimated correctly (27).

BTV and EHDV virus particles are morphologically undistinguishable and have similar biochemical properties. EHDV is composed of 10 double-stranded RNA segments surrounded by two coats of proteins (6). For BTV, it has been shown that the inner capsid exhibits a capsomeric structure and consists of two major proteins, VP3 and VP7, associated with three other minor proteins, VP1, VP4, and VP6. Recent cryoelectron microscopic studies suggested that the BTV core consists of a nucleoprotein center surrounded by two distinct protein layers, each of which may be composed of a single polypeptide species (21a). Immunogold analysis indicated that VP7 is the principal component of the outermost layer and is attached to a framework of VP3 (7). VP7 appeared to be clustered into trimers at all the local and strict three-fold axes (21a). The capsomeric core structure is surrounded by an outer capsid of two major proteins, VP2 and VP5. Identical structural characteristics can be anticipated for EHDV, although less information is presently available.

Considering the biology of the two viruses, a potential for genetic interactions exists. Reoviridae have high capabilities for gene reassortment, playing a role in the generation of genetic diversity and the evolution of the viral strains. The role of reassortment between EHDV and BTV in the generation of genetic diversity is still unknown. Until now, reassortants have been isolated (in vitro and in vivo) within BTV and EHDV strains but never between BTV and EHDV (1), suggesting the potential existence of an exclusion phenomenon. In fact, little is known about the genetic relationship between the two viruses, and of the 10 RNA segments, weak or no cross-hybridization was found for segments 1, 3, and 7 (encoding, respectively, VP1, VP3, and VP7), depending on the stringency conditions (1, 4, 24, 25).

It was therefore of interest to study the relationship between EHDV and BTV in terms of protein-protein interaction. To this end, a functional assay was developed to determine whether the structural proteins could be exchanged between the two orbiviruses.

In 1989, French et al. demonstrated the synthesis of BTV single-shelled corelike particles by using a dual recombinant baculovirus expressing VP3 and VP7 (3), and the structures formed in insect cells were shown to be similar to authentic BTV cores in terms of size, appearance, and stoichiometric arrangement of the two proteins. Moreover, in 1990, the synthesis of double-shelled viruslike particles (VLPs) was achieved by the simultaneous expression of VP3, VP7, VP2, and VP5 (2). To study the relatedness of EHDV and BTV, the recently cloned (29) EHDV-1 VP3 gene was expressed

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by using baculovirus vectors. This report describes the expression of the EHDV-1 VP3 protein as well as the characterization of chimeric structures between EHDV and BTV. The sequence of the EHDV-1 VP3 protein was deduced from the nucleotide sequence of the L3 gene, and the genetic relationship between EHDV and BTV, concerning at least this particular protein, is discussed.

MATERIALS AND METHODS

Viruses and cells. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the recombinant viruses were propagated in *Spodoptera frugiperda* (IPLB-SF21) cells at 28°C, using TC100 medium (GIBCO International) supplemented with 10% fetal calf serum as described by Possee et al. (20, 21).

DNA manipulations and construction of DNA clones. Plasmid DNA manipulations were done essentially as summarized by Maniatis and associates (13) and Perbal (19). Restriction enzymes, T4 DNA ligase, *Bal*31 nuclease, and the Klenow large fragment of DNA polymerase I were purchased from Amersham International plc. Calf intestinal alkaline phosphatase was obtained from Boehringer GmbH (Mannheim, Germany).

Construction of an AcNPV recombinant transfer vector. The construction of recombinant plasmid pAcEHDV1-3 is summarized in Fig. 1. The 2.9-kb fragment containing the complete VP3 gene was recovered from plasmid pEHD1-3/A16 (29) by digestion with *Pst*I, and the terminal dC-dG sequences introduced during the cDNA cloning were removed by *Bal*31 exonuclease. The DNA product was repaired with the Klenow large fragment of DNA polymerase I and ligated into plasmid pUC4K (Pharmacia), previously digested with *Sal*I, repaired with Klenow enzyme, and dephosphorylated. The recombinant plasmids were characterized by appropriate restriction enzyme mapping and dideoxy sequence analysis of the double-stranded plasmid DNA (26). One of these recombinant plasmids, pUC4-EHD1-3, had all of the terminal dC-dG sequences removed at the 5′ end of the gene. The VP3 insert was recovered from pUC4-EHD1-3 by partial *Bam*HI digestion and ligated to the *Bam*HI site of the dephosphorylated transfer vector pAcYM1 (15). The derivative recombinant transfer vector (pAcEHD1-3) containing the EHDV1-3 gene under the control of the polyhedrin promoter was characterized by restriction mapping. The 3′ and 5′ sequences at the insertion site were determined by the plasmid sequencing method (26) to confirm the orientation of the VP3 coding region and the retention of the translation initiation codon.

Transfection and selection of recombinant virus AcEHD1-3. *S. frugiperda* cells were transfected with a mixture of plasmid pAcEHD1-3 DNA (25 μg) and wild-type AcNPV DNA (1 μg) as described by Matsuura et al. (15). The recombinant baculovirus AcEHD1-3 was selected by virtue of its polyhedrin-negative phenotype and was subsequently plaque purified as described previously (15).

Protein analyses. Confluent monolayers of *S. frugiperda* cells in 35-mm tissue culture dishes were infected with virus at a multiplicity of infection (MOI) of 5 PFU per cell. After infection, the medium was removed from the dishes. The monolayers were rinsed three times with phosphate-buffered saline (PBS), and the cells were lysed in 150 μl of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.5 M NaCl, 0.05 M Tris-HCl, 0.01 M EDTA, 0.1% sodium dodecyl sulfate [SDS], pH 7.4). Aliquots of the protein samples were boiled for 10 min in dissociation buffer (2.3% SDS, 10% [vol/vol] glycerol, 5% [vol/vol] β-mercaptoethanol, 62.5 mM Tris-HCl, 0.01% bromophenol blue, pH 6.8), and proteins were resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE) and stained with Kenacid blue (18).

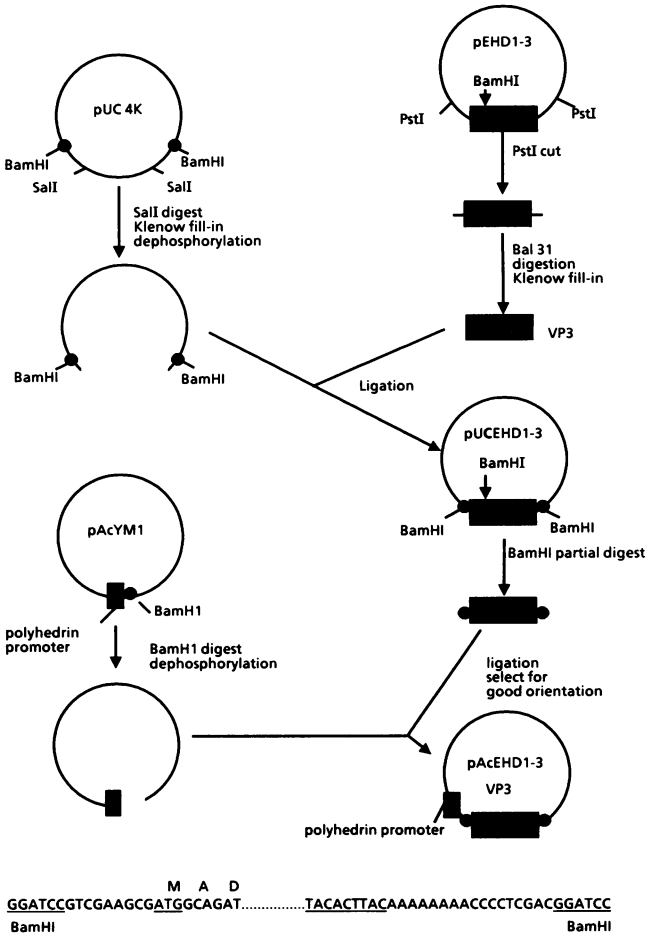

FIG. 1. Schematic diagram of the construction of the plasmid transfer vector pAcEHD1-3. The EHDV-1 VP3 gene was recovered from the cloning vector pEHD1-3/A16 by digestion with *Pst*I followed by digestion with *Bal*31. The DNA product was subcloned in pUC4K to yield plasmid pUCEHD1-3, digested with *Bam*HI, and inserted into the transfer vector pAcYM1 (15), producing the recombinant plasmid pAcEHD1-3 with the EHDV-1 VP3 gene under the control of the polyhedrin promoter. The orientation of the insert was determined by restriction fragment analysis and confirmed by sequencing. The sequence shown at the bottom begins with the *Bam*HI site of the transfer vector (15) and is followed by the sequence through the methionine (M), alanine (A), and aspartic acid (D) codons at the beginning of the EHDV-1 VP3 gene. The sequence shown finishes with the 3′ consensus sequence of the segment 3 followed by the *Bam*HI site.

Immunoblotting analyses. Proteins resolved by SDS-PAGE were electroblotted to an Immobilon membrane (Millipore International). The blot was incubated for 1 h in the blocking solution (5% [wt/vol] milk powder and 0.1% Nonidet P-40 [NP-40] in PBS), then transferred to a blocking solution containing the appropriate polyclonal antiserum (mouse anti-EHDV-1 VP3, mouse anti-BTV-10 VP2, rabbit anti-EHDV-1, or rabbit anti-BTV-2, -10, -11, -13, or -17), and incubated for 1 h. After three washes for 10 min in the same buffer, the bound antibody was detected by using an

appropriate alkaline phosphatase-conjugated immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.); 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Gibco, BRL International) were used as substrates for the alkaline phosphatase.

Production of antibodies in mice. *S. frugiperda* cells were infected with AcEHD1-3 at an MOI of 5 PFU per cell. After 3 days of incubation at 28°C, the cells were harvested, rinsed with PBS, and resuspended in PBS at a concentration of 10^7 cells per ml. Cells were disrupted by three freeze-thaw cycles and kept at −20°C. Mice were injected intramuscularly and intraperitoneally, each with 0.5×10^7 cells per week for 3 weeks. The antigen was injected in 10% (vol/vol) aluminum hydroxide adjuvant (a generous gift from Virbac Laboratory, Carros, France). The sera were collected 1 week after the last injection.

Purification of particles. *S. frugiperda* cells were coinfected at an MOI of 5 PFU per cell with each of the recombinant baculoviruses. Cells were harvested 72 h postinfection, washed with PBS, and lysed at 4°C in TNN buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% NP-40). To verify the simultaneous expression of the proteins, dissociation buffer was added to an aliquot of each sample and the proteins were resolved by SDS-PAGE. The expressed particles were purified by banding either on a discontinuous sucrose gradient (30 to 50% [wt/vol] in 0.2 M Tris-HCl, pH 8.0) after centrifugation at 85,0000 × *g* for 3 h or on self-forming CsCl (35%) density gradients after centrifugation at 140,000 × *g* for 18 h. The particles were analyzed by SDS-PAGE or examined by electron microscopy. BTV cores and particles were purified from monolayers of BHK-21 cells infected with BTV serotype 10 as previously described (16).

Electron microscopy. Purified particles were adsorbed onto copper 400-mesh Formvar carbon-coated grids by floating the grids on droplets of the material for 2 min. After being washed twice in 0.2 M Tris-HCl (pH 8.0), the particles were stained for 20 s in droplets of 2% uranyl acetate. All grids were examined in a JEOL electron microscope.

Sequencing. Four overlapping fragments of L3 cDNA clone of EHDV-1 were isolated by restriction enzyme digestion of the transfer vector pAcEHD1-3. Each fragment (*Bam*HI [3]-*Bam*HI [488], *Bam*HI [489]-*Eco*RV [1207], *Eco*RV [1208]-*Eco*RI [2039], and *Eco*RI [2040]-*Bam*HI [2787]) was repaired with the Klenow fragment of DNA polymerase, purified, and ligated into pUC118 (Pharmacia), previously digested with *Sma*I and dephosphorylated. Recombinant plasmids were isolated by appropriate restriction enzyme mapping. The orientation of the inserts in the recombinant plasmids was determined by using the Sequenase version 2.0 DNA sequencing kit from U.S. Biochemical Corp. (Cleveland, Ohio). Subclones possessing the insert in both orientations were selected. Single-stranded DNA was prepared from these eight subclones as described by Perbal (19); the fragments were then sequenced by using [^{35}S]dATP and 6 and 4% polyacrylamide-urea gels.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence data is M61879.

RESULTS

Construction of the recombinant transfer vector and selection of recombinant baculoviruses. The L3 segment was recovered from the original cloning vector by *Pst*I digestion (Fig. 1; Materials and Methods). The homopolymeric dG-dC tails were removed by *Bal*31 exonuclease to reduce the length of the 5′ noncoding sequence, since this can lead to suboptimal expression by the AcNPV recombinant baculovirus (15). The DNA product was then transferred to a pUC4K vector to add a *Bam*HI site at each extremity, recovered, and inserted into the pAcYM1 baculovirus transfer vector, under the control of the AcNPV polyhedrin promoter (15). The correct orientation of the insert in pAcEHD1-3 and the sequence between the ATG of the L3 gene and the polyhedrin transcription initiation site were determined by dideoxynucleotide sequence analyses (Fig. 1). As shown in Fig. 1, the open reading frame of the L3 gene was flanked at its 5′ extremity by nine nucleotides, including five additional nucleotides derived from pUC4K polylinker. At its 3′ terminus, the L3 segment was complete, containing a noncoding sequence of nine nucleotides (5′-....TACACTTAC-3′) identical to the consensus found at the 3′ terminus of the BTV RNA segments (23; see Fig. 8). Sequencing data from the original clone pEHD1-3/A16 showed that the EHDV-1 L3 segment had a 5′ noncoding sequence of 17 nucleotides starting with a GTTAAA, corresponding to the BTV RNA segment 5′ consensus (23; see Fig. 8).

In the recombinant transfer vector pAcEHD1-3, the sequence flanking the ATG that initiates the VP3 protein open reading frame was GAAGCGATGGCAG. The G at the −3 position conforms to the consensus sequence of Kozak (9–11).

S. frugiperda cells were transfected with mixtures of AcNPV DNA (1 μg) and pAcEHD1-3 DNA (25 μg) as described by Matsuura et al. (15). Plaques produced by the progeny viruses from the transfection were screened, and recombinants exhibiting a polyhedrin-negative phenotype were selected (frequency of ca. 1%). After replaquing three times, five putative recombinants were obtained. No difference in the phenotypes of the viruses in terms of the virus-induced protein sizes or amounts synthesized were detected. One of the recombinants (AcEHD1-3) was chosen for further analyses.

Expression of the VP3 core protein in *S. frugiperda* insect cells. Confluent monolayers of *S. frugiperda* cells were mock infected or infected at an MOI of 5 PFU per cell with either AcNPV or AcEHD1-3. For comparison, cells were also infected with the recombinant baculovirus AcBTV17-3 expressing VP3 from BTV-17 (8). The proteins were resolved by SDS-PAGE (Fig. 2A) and analyzed by Western immunoblotting (Fig. 2B). As expected, no trace of the 29-kDa polyhedrin protein was detected in the AcEHD1-3-infected insect cells (Fig. 2A). In contrast to the mock-infected and AcNPV-infected cell lysate, a high-molecular-weight protein with an estimated size of 103 kDa was detected in the AcEHD1-3-infected cells (Fig. 2A). This protein also comigrated with the VP3 protein from purified EHDV-1 virions and the expressed BTV-17 VP3 protein (Fig. 2A). This agreed with the size of the VP3 proteins reported for EHDV and BTV purified virions (17, 28). Confirmation that the expressed protein represented authentic EHDV polypeptide was provided by Western blot analyses with an anti-EHDV serum (Fig. 2B). This antiserum reacted strongly with the VP3 protein in AcEHD1-3-infected *S. frugiperda* cells, while no reaction was detected with mock-infected or AcNPV-infected cells. Conversely, a monospecific antiserum raised against the expressed VP3 protein was able to bind and to recognize the VP3 protein of EHDV virions (Fig. 2C). Neither the preimmune mouse antiserum nor the control serum raised to AcNPV-infected insect cells recognized this protein (data not shown).

The AcEHDV1-3-infected *S. frugiperda* cells were har-

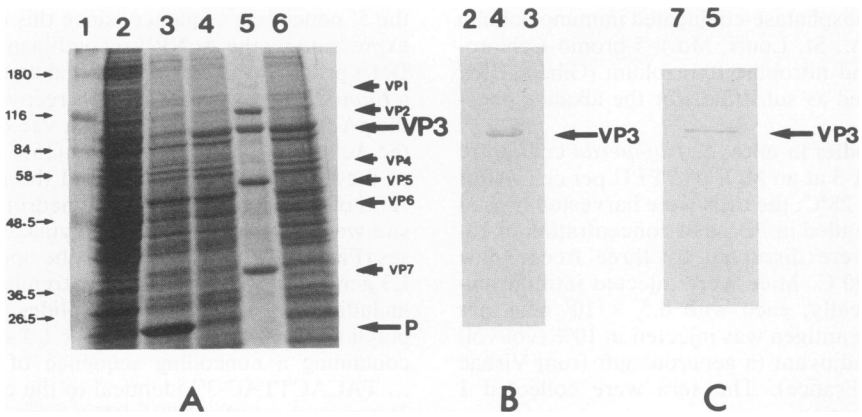

FIG. 2. Expression of the EHDV-1 VP3 protein in insect cells by a recombinant baculovirus and confirmation of its authenticity by Western immunoblot analysis. *S. frugiperda* cells were infected at an MOI of 5 PFU per cell with the recombinant baculovirus expressing the EHDV-1 VP3 protein (lane 4) or the recombinant baculovirus expressing the BTV-17 VP3 protein (lane 6), and the cells were harvested 3 days postinfection. Mock-infected *S. frugiperda* cells (lane 2) and wild-type AcNPV-infected *S. frugiperda* cells (lane 3) served as controls. Authentic EHDV virions prepared from EHDV-infected BHK cells (lane 5) as well as mock-infected BHK cells (lane 7) were included for comparison. Proteins recovered from the cells extracts were separated by SDS-PAGE and stained with Kenacid blue (A) or resolved by gel electrophoresis and analyzed by Western blotting using either an anti-EHDV polyclonal serum (B) or an anti-EHDV-1 VP3 monospecific antiserum (C) (see Materials and Methods). Molecular weight markers (lane 1) were also included; sizes are indicated in kilodaltons. Positions of the AcNPV polyhedrin protein (P) and the EHDV-1 VP3 protein are indicated.

vested at different time points and lysed, and the cells extracts were resolved by SDS-PAGE and stained with Kenacid blue. The maximum VP3 protein expression was obtained 3 days postinfection (Fig. 2A), representing 30% of total cellular protein synthesis as estimated by scans of the stained protein profiles (data not shown). This represented an amount similar to that obtained for BTV-17 VP3 protein. By comparison with a parallel electrophoresis containing known quantities of bovine serum albumin, it was estimated that the level of VP3 protein expression was 40 mg/liter of 2×10^9 AcEHD1-3-infected *S. frugiperda* cells.

Formation of chimeric corelike particles between EHDV-1 and BTV-10. An attempt was made to investigate whether VP3 of EHDV could replace VP3 of BTV and form chimeric corelike particles. *S. frugiperda* cells were therefore coinfected with AcEHD1-3 and with the recombinant baculovirus expressing BTV-10 VP7 (AcBTV10-7) (17). As a control, insect cells were coinfected at the same multiplicity with the recombinant baculovirus expressing BTV-17 VP3 (AcBTV17-3) and AcBTV10-7. Three days postinfection, the cells were lysed with the nonionic detergent NP-40 and structures were purified on discontinuous sucrose gradient as described previously. Examination by electron microscopy revealed empty corelike particles similar in size and appearance to BTV corelike particles prepared in the same conditions (Fig. 3A and B). However, the chimeric corelike particles seemed more heterogeneous than the BTV corelike particles. The arrangement of the VP7 capsomeres in the core outer layer appeared more or less dense and regular. In any case, the VP3 inner layer of the core was conserved, exhibiting an icosahedral configuration (55 nm in diameter). This was probably due to the lack of stability of the chimeric corelike particles during the fixative treatments required for electron microscopy. No subcores of VP3 could be observed in any of the preparations (i.e., BTV or chimeric corelike particles) in these conditions. Nevertheless, after dialysis in 0.2 M Tris (pH 8.0), the chimeric cores were shown to be as stable as BTV corelike particles and were intact after at least 4 weeks at 4°C.

When purified on a CsCl gradient, the chimeric particles were very unstable and a small amount of particles was recovered compared with BTV corelike particles prepared in the same conditions (data not shown). Under the electron microscope, few complete corelike particles were observed; most of them had lost their capsomeres, and the inner subcores were also damaged in some cases (data not shown).

To determine their protein composition, the purified particles were analyzed by SDS-PAGE and shown, after scanning of the stained protein profile, to contain both VP3 and VP7 in the same proportions as in BTV corelike particles (Fig. 4).

Assembly of double-shelled chimeric VLPs. To investigate further the authenticity of the chimeric corelike particles, *S. frugiperda* cells were coinfected (MOI of 5 PFU per cell for each virus) with the two recombinant viruses described above and the dual recombinant baculovirus expressing VP2 and VP5, the two outer capsid proteins of BTV (2). After 2 days of infection, particles were purified as described in Materials and Methods. Examination by electron microscopy revealed empty double-shelled particles comparable to BTV VLPs previously described (2) (Fig. 5). The presence of an outer capsid resulted in the loss of the capsomeres characteristic of the corelike particles. In both preparations, a range of intermediate structures was observed, with various amounts of outer capsid proteins attached to the cores, from corelike particles with a diameter of 65 nm to VLPs with an estimated diameter of 85 nm. The protein composition of these particles was then checked by SDS-PAGE and Western blot analyses. However, because of the heterogeneity of the VLPs in terms of outer capsid size, no stoichiometric analysis of the VP2 and VP5 proteins was feasible. On the Kenacid blue-stained gel, another protein species corresponding to VP5 in the BTV VLPs was detected in the chimeric VLPs (Fig. 6A). The authenticity of VP5 was then confirmed by Western blot analyses using an anti-BTV-10 antiserum (Fig. 6B). As VP3 and VP2 comigrated, the presence of VP2 was demonstrated by Western blot analyses using an anti-VP2 specific antiserum (Fig. 6C).

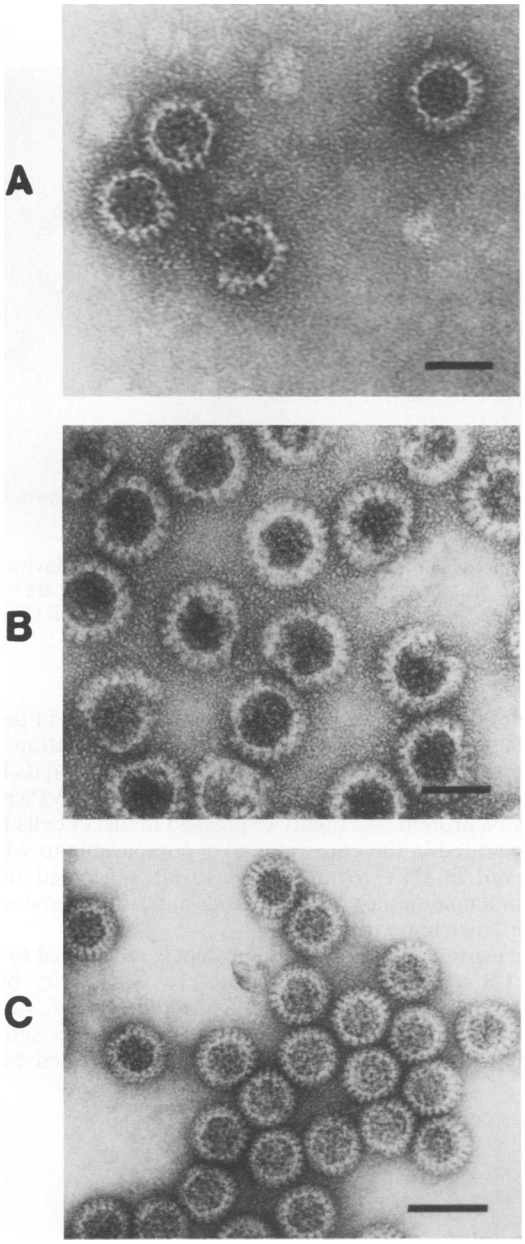

FIG. 3. Electron micrographs of empty EHDV chimeric corelike particles synthesized in insect cells by coexpression of the EHDV-1 VP3 and the BTV-10 VP7 core proteins with use of baculovirus vectors. Expressed chimeric corelike particles purified on discontinuous sucrose gradients (A) are compared with BTV corelike particles prepared in the same conditions (B). Authentic BTV core particles purified on CsCl gradients are included for comparison (C). Bars, 100 nm.

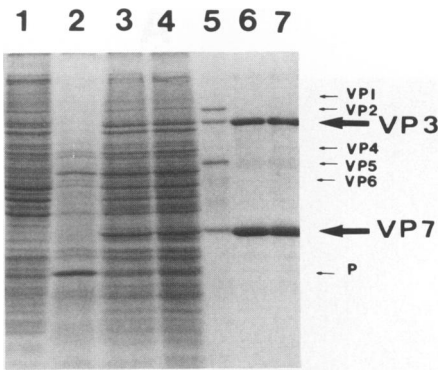

FIG. 4. Protein analysis of the purified chimeric corelike particles. *S. frugiperda* cells were coinfected with the recombinant baculovirus expressing EHDV-1 VP3 core protein and the recombinant baculovirus expressing BTV-10 VP7 core protein (MOI of 5 PFU per cell for each virus). As a control, cells were coinfected at the same MOI with the recombinant baculovirus expressing BTV-17 VP3 protein and the recombinant baculovirus expressing BTV-10 VP7 protein. Cells were harvested 72 h postinfection and lysed with the nonionic detergent NP-40, an aliquot of the cell lysate was kept to check the simultaneous expression of the two proteins, and the expressed particles were purified on discontinuous sucrose gradients. The protein samples were resolved by SDS-PAGE and stained with Kenacid blue. Mock-infected (lane 1) and wild-type AcNPV-infected (lane 2) *S. frugiperda* cells served as controls. EHDV virions purified from infected BHK cells were included for comparison (lane 5). BTV cell lysate and BTV purified corelike particles are shown in lanes 3 and 6, respectively. EHDV cell lysate and EHDV chimeric corelike particles are shown in lanes 4 and 7, respectively.

Interaction of EHDV-1 VP3 and BTV-17 VP3 proteins with the three minor core proteins, VP1, VP4, and VP6. The incorporation of BTV-10 VP1 into BTV corelike particles has been reported recently by Loudon and Roy (12), using coinfections of *S. frugiperda* cells with the dual recombinant baculovirus expressing VP3 and VP7 of BTV (AcBTV17.3/BTV10.7) and the recombinant baculovirus expressing VP1 from BTV10 (AcBTV10-1). Assays to incorporate the BTV VP1 protein simply by mixing a lysate of *Spodoptera* cells infected with AcBTV17.3/BTV10.7 with a lysate of *Spodoptera* cells infected with AcBTV10-1 were unsuccessful, as were attempts to incorporate a protein involved in transcription and replication such as the rabies virus N protein into BTV corelike particles, using the same technology (data not shown). These results demonstrated that the VP1 protein was incorporated during the corelike particles' morphogenesis and that specific interactions between the VP1 protein and the VP3 inner core protein were probably involved in this process. Nevertheless, an initial attempt using the same experimental approach to incorporate VP4 and VP6 into the corelike particles was unsuccessful.

To study the relationship between VP3 from BTV and EHDV in terms of protein structure and function, similar experiments were undertaken with VP3 from EHDV-1. To increase the efficiency of the coinfections, a dual recombinant baculovirus expressing VP3 from EHDV-1 and VP7 from BTV-10 was required. This dual recombinant baculovirus (AcEHD1-3/BTV10-7) was constructed similarly to the BTV dual recombinant baculovirus described by French et al. (3) (data not shown). Subsequently, *S. frugiperda* cells were coinfected at an MOI of 5 PFU per cell with the dual recombinant AcEHD1-3/BTV10-7 and with one of the following recombinant baculoviruses: AcBTV10-1 (VP1), AcBTV10-4 (VP4), or AcBTV10-9 (VP6). Similar experiments were undertaken with the BTV dual recombinant baculovirus AcBTV17-3/BTV10-7 for comparison. After 2 days of infection, when the cells were 100% infected but still 98% viable, the cells were harvested and particles were purified as described in Materials and Methods. The protein contents of the particles purified from the six different coinfections were analyzed by SDS-PAGE (Fig. 7). VP1 could be incorporated in large amounts in BTV corelike

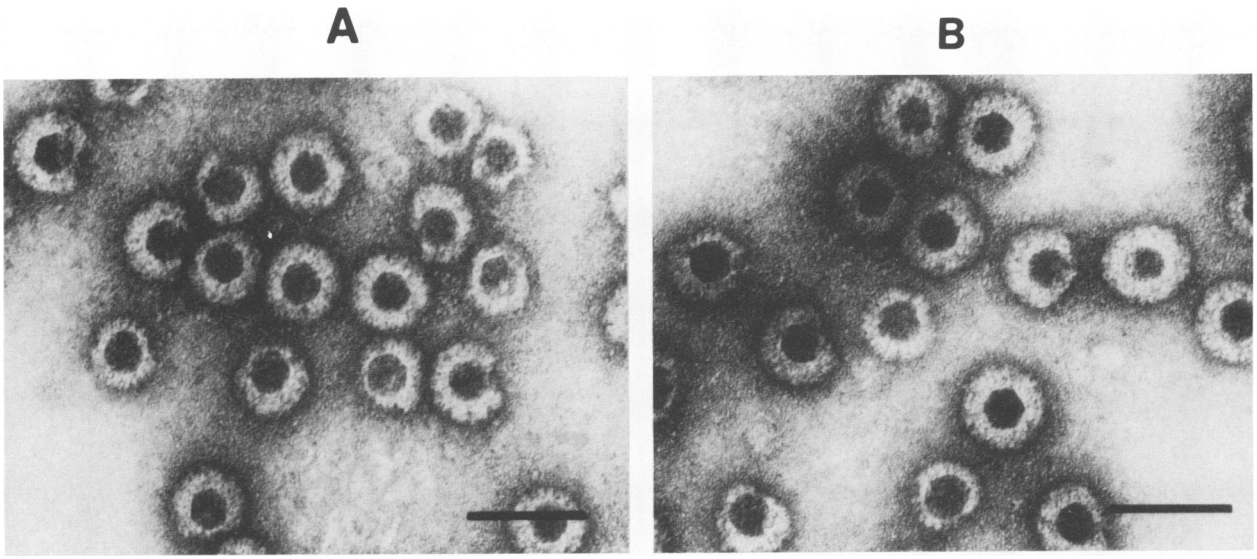

FIG. 5. Electron micrographs of double-shelled EHDV chimeric VLPs synthesized in insect cells by three recombinant baculoviruses: one expressing the EHDV-1 VP3 core protein, one expressing the BTV-10 VP7 core protein, and the dual recombinant expressing BTV-10 VP2 and VP5 capsid proteins. Expressed chimeric particles purified on discontinuous sucrose gradients (A) are compared with BTV corelike particles prepared in the same conditions (B). Bars, 100 nm.

particles, as expected, but also with a similar efficiency in EHDV chimeric corelike particles. In these conditions, VP4 was found in large amounts in BTV corelike particles as well as in EHDV chimeric corelike particles. VP6 was also incorporated in both types of particles but with less efficiency (Fig. 7A and B). In another coinfection experiment with the two viruses, it appeared that the incorporation of the VP6 protein in EHDV chimeric particles was better than with the previous coinfection (Fig. 7C).

The amounts of VP1, VP4, and VP6 proteins found in the corelike particles were comparable to those observed in the BTV virions. Unfortunately, no stoichiometric analysis of the different proteins was feasible, since the maximum efficiency of the coinfections was probably not 100% and therefore empty corelike particles were present in the preparations. In any case, the level of incorporation of the protein in the corelike particles did not depend on its level of expression in insect cells, as demonstrated with VP6 protein. The VP6 protein was highly expressed in insect cells but still incorporated in the cores at a level comparable to what was observed in BTV virions. This result suggested that this phenomenon did not occur at random but was dependent on stoichiometric restraints.

The expressed EHDV-1 VP3 protein is recognized by all five BTV US serotypes. The antigenic cross-reactivity between VP3 from EHDV and BTV was studied by Western blot analyses using anti-BTV sera from the five US serotypes. Chimeric cores were used as a source of purified EHDV-1

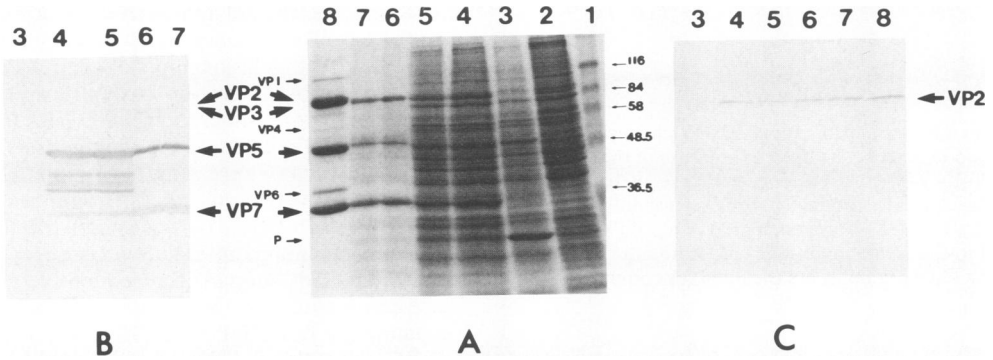

FIG. 6. Protein analysis of the purified double-shelled chimeric VLPs. *S. frugiperda* cells were coinfected with two recombinant baculoviruses (see legend to Fig. 4) and the dual recombinant baculovirus expressing the two outer capsid proteins, VP2 and VP5, of BTV-10 (MOI of 5 PFU per cell for each virus). The cells were harvested 72 h postinfection, and particles were purified as described in the legend to Fig. 4. The protein samples were resolved by SDS-PAGE and stained with Kenacid blue (A) or analyzed by Western blotting using an anti-BTV-10 polyclonal serum (B). Since VP2 comigrated with VP3, its presence in the purified double-shelled VLPs was confirmed by reaction with a monospecific anti-BTV-10 VP2 serum (C). Molecular weight markers are shown in lane 1; sizes are indicated in kilodaltons. Mock-infected (lane 2) and wild-type AcNPV-infected (lane 3) *S. frugiperda* cells served as controls. BTV-10 purified virions were included for comparison (lane 8). BTV cell lysate and purified VLPs are shown in lanes 4 and 6, respectively. EHDV cell lysate and purified chimeric VLPs are shown in lanes 5 and 7, respectively. Panel B shows three extra reactive bands below VP5 in lanes 4 and 5 corresponding to breakdown products of VP5 in *S. frugiperda* cells as described previously by Marshall and Roy (14).

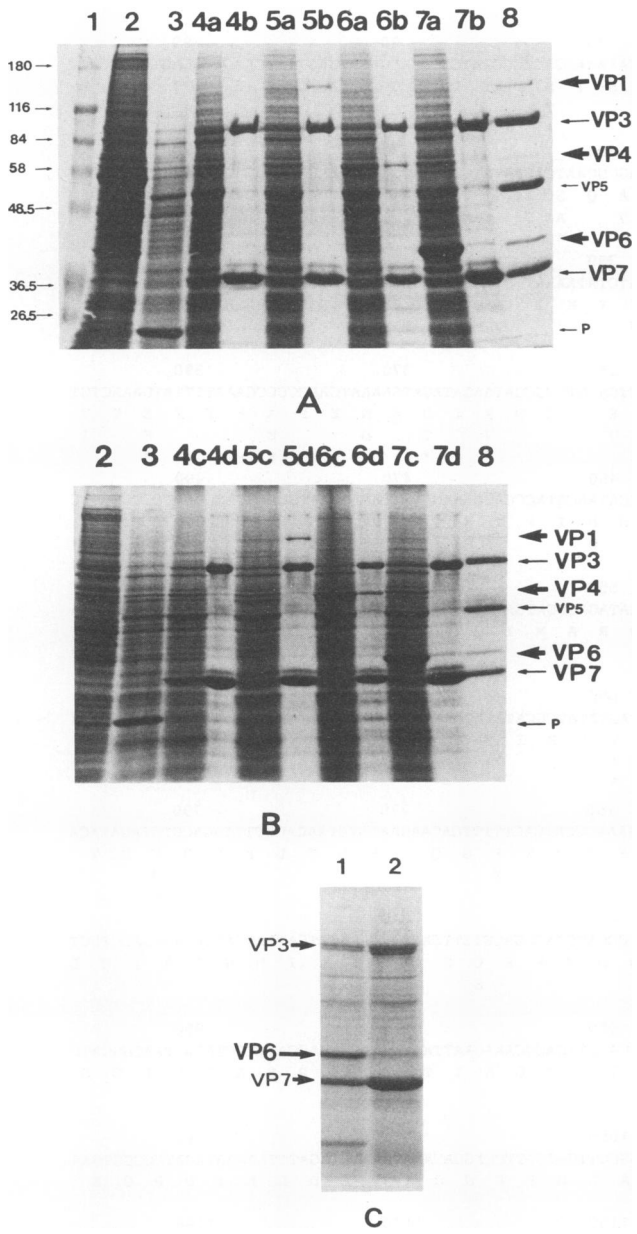

FIG. 7. Incorporation of the three minor core proteins VP1, VP4, and VP6 in BTV and EHDV corelike particles. *S. frugiperda* cells were coinfected either with the dual recombinant baculovirus expressing the EHDV-1 VP3 and BTV-10 VP7 core proteins (A and C) or with the dual recombinant virus expressing the BTV-17 VP3 and BTV-10 VP7 core proteins (B), together with one of the recombinant baculoviruses expressing either VP1, VP4, or VP6 of BTV-10. The cells were harvested 2 days postinfection, and corelike particles were purified as described in the legend to Fig. 4. The protein samples were resolved by SDS-PAGE and stained with Kenacid blue. (A) EHDV-BTV chimeric corelike particles. Lanes: 1, molecular weight markers (sizes indicated in kilodaltons); 2, mock-infected *S. frugiperda* cells; 3, wild-type AcNPV-infected *S. frugiperda* cells; 4 to 7, cell lysate (a) and purified corelike particles (b) alone (lanes 4a and 4b) containing VP1 (lanes 5a and 5b), containing VP4 (lanes 6a and 6b), and containing VP6 (lanes 7a and 7b); 8, authentic BTV particles. (B) Corelike particles containing BTV VP3 and VP7. Lanes: 2, 3, and 8, as above; 4 to 7 cell lysate (c) and purified corelike particles (d) alone (lanes 4c and 4d), containing VP1 (lanes 5c and 5d), containing VP4 (lanes 6c and 6d), and containing VP6 (lanes 7c and 7d). (C) EHDV-BTV chimeric corelike particles. Lanes: 1, particles containing VP7; 2, particles alone.

TABLE 1. Antigenic cross-reactivities between EHDV and BTV VP3 proteins

VP3 origin	Cross-reactivity with indicated antiserum				
	BTV-2	BTV-10	BTV-11	BTV-13	BTV-17
EHDV-1 chimeric cores	+	+++	++	++	+++
EHDV-1 virions	+	+++	++	ND[a]	+++
BTV-17 cores	++	+++	+++	++	+++

[a] ND, not determined.

VP3 protein; synthetic BTV cores as well as EHDV-1 purified virions were used as controls. The protein samples were resolved by SDS-PAGE, and after electrotransfer of the proteins to an Immobilon membrane, the blot was treated as described in Materials and Methods, using either anti-BTV-2, -10, -11, -13, or -17 antiserum. As shown in Table 1, the EHDV-1 VP3 protein exhibited the same reactivities as did the original VP3 from EHDV-1 virions and was recognized by all the BTV antisera. The signals detected with the EHDV-1 VP3 protein were similar to those of the BTV17 VP3 protein, i.e., a strong signal with anti-BTV-17, -11 and -10 antisera, whereas a weaker reactivity was found with anti-BTV-2 and -13 antisera. This finding was in agreement with previous results showing antigenic cross-reactivity between VP3 from EHDV-1 and BTV-10 (6).

Sequence homology between EHDV and BTV VP3 proteins. The complete nucleotide sequence of the VP3 gene was determined from a complete cDNA clone, using the dideoxynucleotide technique. The entire sequence is 2,768 nucleotides long, with a single extensive open reading frame coding for a 899-amino-acid protein (Fig. 8). The EHDV-1 VP3 amino acid sequence was compared with the previously published sequences of BTV-10, BTV-17, and BTV-1 (Australian serotype) (4, 5, 22). The positions of the differences between the EHDV-1 sequence and a consensus of three BTV serotypes sequences are indicated in Fig. 8; 22% amino acid changes are present between the two sequences, spread throughout the VP3 gene. Interestingly, 10% of the 22% of changes preserve the functional type of the amino acid (i.e., polar, hydrophobic, or charged). To determine the evolutionary relationship between the two proteins, the distance between the VP3 protein from the four different origins (EHDV-1, BTV-10, BTV-17, and BTV-1) was analyzed by computer, and the results indicated that EHDV was equally distant from all of the BTV serotypes, which were much closer to each other (Table 2).

DISCUSSION

BTV and EHDV, members of the *Orbivirus* genus, were shown to be related (6, 24). Moreover, recent observations on the biology of the two viruses (host spectrum and pathogeny) suggested that EHDV and BTV could interact in vivo (27). However, until now no reassortants between EHDV and BTV could be isolated in vitro (1).

Therefore, we have developed a functional assay to study the relatedness of EHDV and BTV in terms of protein-protein interaction of the EHDV VP3 protein with the other BTV capsid proteins (VP7, VP2, and VP5) as well as with the other minor core proteins (VP1, VP4, and VP6). This work was completed by sequence comparison analysis of the EHDV VP3 gene with the BTV VP3 gene.

A recombinant baculovirus expressing a high level of the EHDV-1 VP3 protein was isolated. The expressed protein

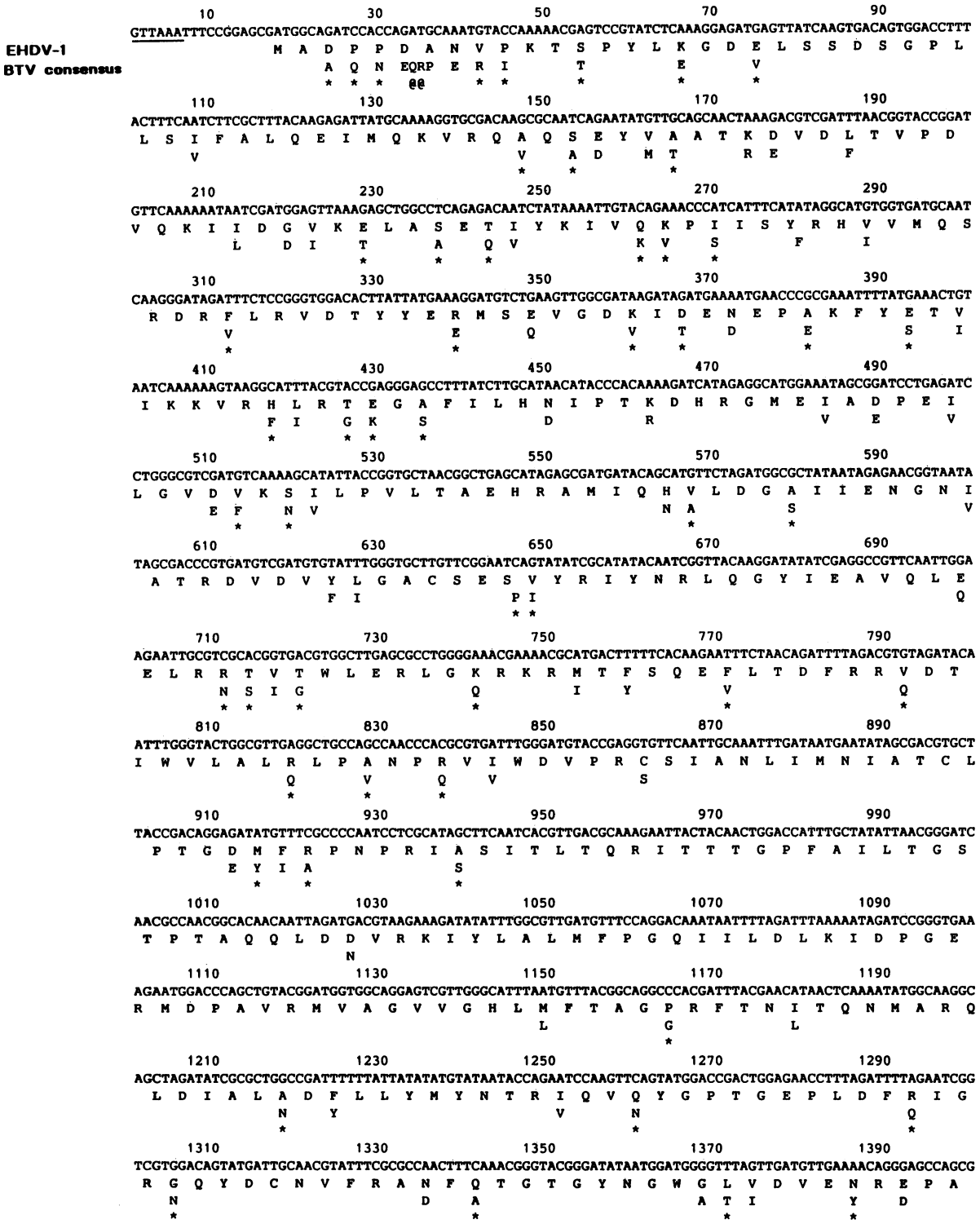

FIG. 8. Nucleotide sequence of the EHDV-1 L3 gene and comparison of the deduced amino acid sequence with those of BTV-10 (21), BTV-17 (4), and BTV-1, Australian serotype (5). The complete nucleotide sequence of the L3 gene of EHDV-1 is presented; the 5′ and 3′ sequences conserved with the BTV segments consensus are underlined. The open reading frame begins at position 18 and is terminated by the TAG codon at position 2715. The deduced amino acid sequence of the EHDV-1 VP3 protein is indicated below the nucleotide sequence. Amino acid changes with the BTV VP3 consensus, determined by using the University of Wisconsin Genetics Computer Group sequence analysis software package, are given below the EHDV-1 VP3 sequence; those that are nonconservative are marked (*). Two extra amino acids present at positions 7 and 8 in the BTV VP3 sequences are also indicated (@@).

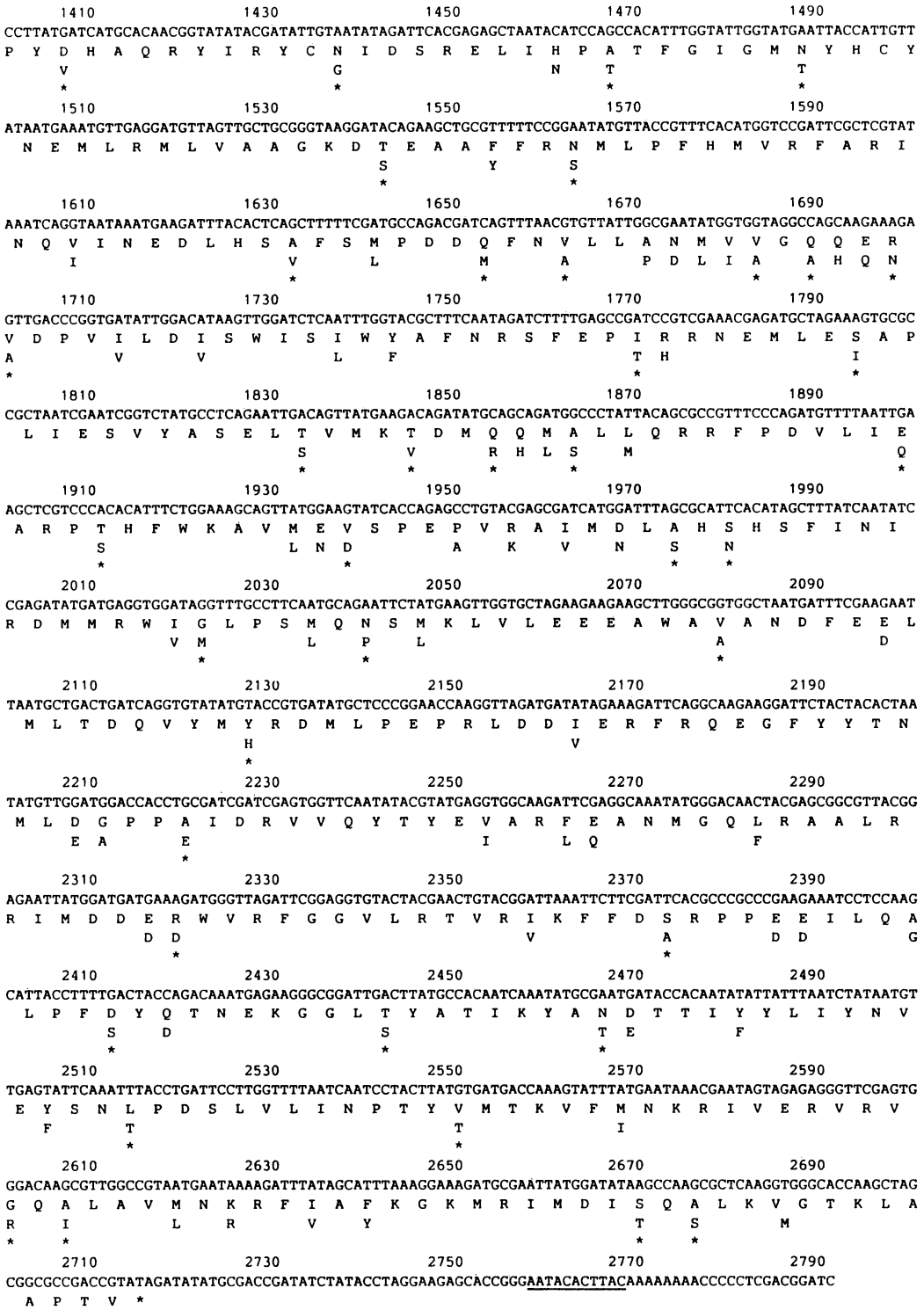

Fig. 8—*Continued.*

was similar to the viral protein in terms of size and antigenic properties, as shown by its reactivity with anti-EHDV serum as well as with anti-BTV sera.

In insect cells, the EHDV-1 VP3 protein was able to interact with the BTV-10 VP7 protein to form chimeric corelike particles identical to BTV core particles in terms of size and arrangement of the VP3 with the VP7 protein. This was confirmed by protein profile scanning of the purified EHDV chimeric corelike particles; the ratio of the VP3 to the VP7 protein was identical to that observed for the BTV corelike particles. The efficiency of EHDV chimeric corelike particle formation was similar to that for BTV corelike

TABLE 2. Distances between EHDV and BTV VP3[a]

Sequence	Distance (%)				
	Consensus	BTV-10	BTV-17	BTV-1	EHDV-1
Consensus	100	100	99.55	99.22	88.85
BTV-10		100	99.45	99.11	89.10
BTV-17			100	98.78	88.65
BTV-1				100	88.43
EHDV-1					100

[a] Distances between the VP3 proteins of EHDV-1, BTV-10 (21), BTV-17 (4), and BTV-1 Australian serotype (5) and a consensus from the four sequences were determined by using the Peptide distances program from the University of Wisconsin Genetics Computer Group sequence analysis software package.

particles, as shown by the amount of particles purified on sucrose gradients from equally expressed EHDV-1 and BTV-17 VP3 proteins. On electron micrographs, the EHDV chimeric corelike particles looked more heterogeneous than the BTV corelike particles; the arrangement of the VP7 capsomeres in the core outer layer appeared more or less dense and regular. However, this was due to the lack of stability of the chimeric corelike particles during the fixative treatments for electron microscopy, as the EHDV and BTV corelike particles exhibited similar protein profiles on a 10 to 50% continuous sucrose gradient (data not shown). The EHDV chimeric corelike particles were very stable at 4°C in 0.2 M Tris (pH 8.0) but were disrupted in a 35% CsCl gradient, as opposed to BTV corelike particles purified in large amounts in the latter conditions. This result demonstrated the conservation of BTV and EHDV VP3 protein structure, regarding at least the interactions with the VP7 protein, the other major component of the cores.

The authenticity of the EHDV chimeric corelike particles was further confirmed in that the BTV-10 VP2 and VP5 proteins could be incorporated on the corelike particles, leading to the assembly of chimeric VLPs similar to BTV VLPs. Moreover, interaction of the EHDV-1 VP3 protein with the three BTV minor core proteins (VP1, VP4, and VP6) was also achieved, since the individual proteins could be incorporated into the EHDV chimeric corelike particles. The stoichiometric analysis of the different proteins in the corelike particles was not feasible in our conditions and will require the construction of baculovirus triple expression vectors.

The conservation of the VP3 proteins was then analyzed at the amino acid level in terms of sequence comparison between EHDV and BTV VP3 genes. The alignment of the EHDV-1 VP3 amino acid sequence with a consensus between the BTV-1, -10, and -17 VP3 sequences revealed 22% amino acid differences distributed throughout the gene. Interestingly, 10% of the changes preserved the functional type of the amino acid (i.e., polar, hydrophobic, or charged). Thus, in the first part of this study, the EHDV and BTV VP3 proteins appeared very conserved in terms of structure and function; the two proteins could be exchanged without any effect on their interactions with the other major and minor BTV core protein components. Moreover, the second part of this work revealed a high degree of conservation (89% at the amino acid level) between EHDV and BTV VP3 genes. This analysis on segment 3 demonstrates the close relationship between EHDV and BTV in the *Orbivirus* phylogeny and indicates the possibility of a common ancestor. In view of the results that we have obtained by using the baculovirus expression system, we should expect to be able to isolate reassortants between EHDV and BTV, at least for the segment 3. Until now, such reassortants have never been described; however, little information is available in the literature (1). We have started mixed infections on BHK-21 cells, using BTV-17 and EHDV-1, and the isolation of reassortants is in progress. This approach could help us to determine whether, in vivo, an exclusion phenomenon really exists between EHDV and BTV.

The BTV core organization model determined from the three-dimensional structure analysis data (21a) indicates that VP3 is the center of the core involved in interactions with VP7 to the outside and with VP1, VP4, VP6, to the inside, and probably with the genomic RNA segments as well. This could lead to a high degree of constraint on the VP3 structure and is correlated with a large degree of restraint in the VP3 variation. This may be why the VP3 gene remained so conserved during the evolution of these two orbiviruses. Little is known, at a molecular level, about the other EHDV structural proteins, but antigenic cross-reactivity was detected for the VP7 protein only, not for the VP2 and VP5 proteins. This indicates that the VP7 proteins are more related than the VP2 and the VP5 proteins, which have differed for a long time as a result of antigenic variation. Similar studies on these other EHDV structural proteins would help to confirm this model.

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