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Identification of Domains in Bluetongue Virus VP3 Molecules Essential for the Assembly of Virus Cores

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Bluetongue virus (BTV) cores consist of the viral genome and five proteins, including two major components (VP3 and VP7) and three minor components (VP1, VP4, and VP6). VP3 proteins form an inner scaffold for the deposition on the core of the surface layer of VP7. VP3 also encapsidates and interacts with the three minor proteins. The BTV VP3 protein consists of 901 amino acids and has a sequence that is a highly conserved among BTV serotypes and other orbiviruses (e.g., epizootic hemorrhagic disease virus and African horse sickness virus). To locate sites of interaction between VP3 and the other structural proteins, we have analyzed the effects of a number of VP3 deletion mutants representing conserved regions of the protein, using as an assay the formation of core-like particles (CLPs) expressed by recombinant baculoviruses. Five of the VP3 deletion mutants interacted with the coexpressed VP7 and made CLPs. These CLPs also incorporated the three minor proteins. One mutant, lacking VP3 amino acid residues 499 to 508, failed to make CLPs. Further mutational analyses have demonstrated that a methionine at residue 500 of VP3 and an arginine at residue 502 were both required for CLP formation.

Virus assembly within infected cells involves a precise sequence of highly specific interactions involving a number of macromolecules (e.g., protein-protein and protein-nucleic acids). A complex nonenveloped RNA virus such as bluetongue virus (BTV) with seven different protein components (VP1 to VP7) offers an attractive subject to study protein-protein interactions that are essential for virus assembly. We have investigated the capsid structure of BTV, an arthropod-borne member of the *Orbivirus* genus (*Reoviridae* family). BTV particles have a diameter of 810 Å (1 Å = 0.1 nm) and are composed of two sets of proteins (8, 20, 27, 28, 30) surrounding a genome of 10 double-stranded RNA segments (L1 to L3, M4 to M6, and S7 to S10). The outer protein shell contains two major proteins species (VP2 and VP5 [30]). When removed, it reveals a core particle that has a diameter of 680 Å and is divided into two concentric layers of protein that enclose the inner components (RNA genome and minor proteins VP1, VP4, and VP6). The surface of the core is composed of 780 molecules of a 38-kDa VP7 protein arranged as 260 trimers (23). These trimers are specifically positioned on an inner layer of 60 copies of a second major protein, VP3, which has a molecular mass of 110 kDa and is arranged in 12 pentamers (7, 23).

We have previously reported that the simultaneous expression of VP3 and VP7 proteins results in the assembly of core-like particles (CLPs) (6). Coexpression of these two proteins in the presence of either VP1 and/or VP4 and/or VP6 leads to the encapsidation of the minor proteins (15, 18). By dialysis, VP7 can be removed from CLPs to yield subcores composed only of VP3 (18), and including the minor protein(s) when they are present, indicating that these proteins also interact with VP3 (15, 18).

On the basis of the knowledge of the composition of cores and CLPs, we have developed a functional assay to identify the regions of VP3 (or VP7) necessary for CLP formation and the incorporation of the minor proteins. Our studies on VP3 have involved the synthesis of a series of internal deletion mutants of the protein and the coexpression of these mutant VP3 proteins in the presence of other proteins of the core (14, 21, 25, 29). From these studies, a region of VP3 that appears to be essential for CLP formation has been identified. Further site-directed mutageneses have identified two amino acids in this region that are required for the synthesis of CLPs.

MATERIALS AND METHODS

Viruses and cells. *Spodoptera frugiperda* cells were grown in suspension or monolayer cultures at 28°C in TC100 medium supplemented with 10% fetal calf serum. *Autographa californica* nuclear polyhedrosis virus and recombinant derivatives were plaque purified and propagated in *S. frugiperda* cells as described elsewhere (2).

Deletion mutations of the BTV VP3 gene and construction of recombinant transfer vectors. Six regions of the L3 cDNA (VP3 gene) of BTV-17 were initially identified for deletion mutagenesis. These represented amino acids (aa) 31 to 40, 345 to 350, 371 to 385, 499 to 508, 526 to 538, and 828 to 837 (Fig. 1). The L3 cDNA in the plasmid transfer vector pAcRP1.17.3 (9, 17) was excised by digestion with *Sma*I and transferred into pAcYM1 (19) to give pAcYM1.17.3. Appropriate restriction fragments were used to excise sequences from pAcYM1.17.3 for replacement with a mutant DNA synthesized by PCR and other manipulations (3, 31). The nucleotide residues identified below for the primers and restriction sites correspond to the published BTV-17 VP3 sequence (24).

For the deletion construct D1 lacking sequences corresponding to aa 31 to 40 (nucleotides 108 to 137), the recombinant transfer vector pAcYM1.17.3 was initially digested with *Nae*I (a site in the plasmid upstream of the VP3 coding sequence) and *Pst*I (nucleotides 241 to 246). The large DNA fragment containing the cut VP3 gene was recovered following

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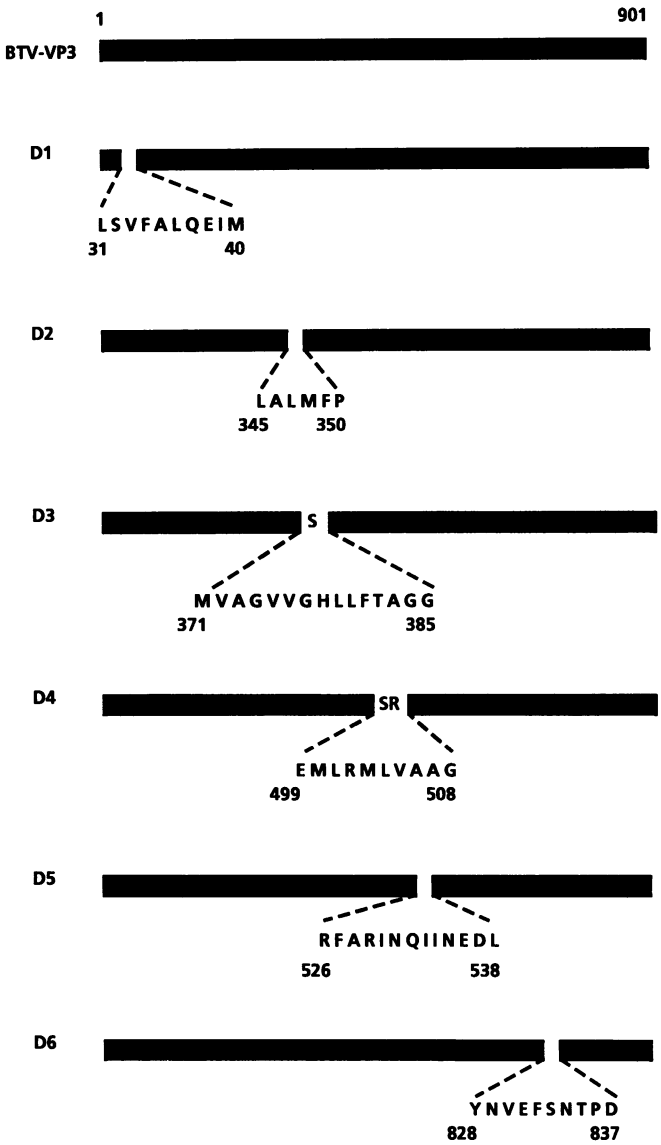


FIG. 1. Diagrams of VP3 deletion mutants D1 to D6. The diagram at the top represents the 901-aa, unmodified BTV-17 VP3. The other solid bars represent the deletion mutants of VP3 that were prepared. The gap in each bar represents the site of the deletion. The amino acid residues removed are indicated below the gaps. Where additional amino acids were inserted in lieu of the deletion, they are indicated (S, SR; see text).

agarose gel electrophoresis. A mutant DNA lacking the D1 target sequences was synthesized by PCR using pAcYM1.17.3 and the appropriate forward and reverse sense primers (Table 1). The protocol to make D1 involved synthesizing a PCR product that included the protein sequence immediately upstream of the deletion site (primers 1 and 2) and a second PCR product that included the protein sequence immediately downstream (primers 3 and 4). Since the products were also designed to have overlapping sequences, they were recovered, pooled, melted, and used with primers 1 and 4 to obtain a single PCR product lacking the required sequences. Primer 1 corresponded to a sequence upstream of the *Nae*I restriction site (Table 1). Primer 2 corresponded to the VP3 nucleotides c147 to c138 and c107 to c88 (where c represents the complement of the VP3 coding-strand nucleotide). Primer 3 corresponded to VP3 residues 98 to 107 and 138 to 156, while primer 4 corresponded to residues c257 to c239. The derived hybrid PCR product was digested with *Nae*I and *Pst*I and ligated to the large VP3 DNA fragment described above to generate the mutant transfer vector pAcYM1.17-3.D1.

For D2 (aa 345 to 350, nucleotides 1050 to 1067), pAc YM1.17-3 was used as the template with forward primer 5 (TG; 1042 to 1049 and 1068 to 1083) and back primer 6 (c1394 to c1375). The mutant PCR product was recovered and then annealed to pAcYM1.17-3 previously digested with *Bgl*II (residues 1042 to 1047) and *Nru*I (1345 to 1350) to generate pAcYM1.17-3.D2.

For D3 (aa 371 to 385, nucleotides 1128 to 1172), pAc YM1.17-3 was used as template for forward primer 7 (239 to 257) and back primer 8 (ATTCTAGA; c1127 to c1109) and used to make a PCR product corresponding to the sequence upstream of the deletion site. Forward primer 9 (TGTCT; 1173 to 1191) and back primer 6 were used to make the downstream sequence. Since both PCR products contained a new *Xba*I site in lieu of the deleted sequences, the two products were recovered, digested with *Xba*I, and ligated, and the hybrid was recovered, redigested with *Pst*I and *Nru*I, and ligated to the *Pst*I- and *Nru*I-cut pAcYM1.17-3 sequence to generate recombinant transfer vector pAcYM1.17-3.D3. Since an additional TCT was inserted into the sequence in lieu of the deleted region, an additional serine (S) was incorporated into the sequence (Fig. 1).

For D4 (aa 499 to 508, nucleotides 1512 to 1541), forward primer 9 and back primer 10 (TTTCTAGA; c1511 to c1493) were used with pAcYM1.17.3 to make an upstream PCR product, and forward primer 11 (ATTCTAGA; 1542 to 1558) and back primer 12 (c1745 to c1730) were used to make a downstream PCR product. By design, both products contained *Xba*I sites in the overlap region. Consequently they were recovered, mixed, treated with *Xba*I, and ligated, and the full-length product was recovered and digested with *Eco*RV (1736 to 1741) and *Nru*I to remove the extremities before ligation to *Eco*RV- and *Nru*I-cut pAcYM1.17.3 to generate the mutant transfer plasmid pAcYM1.17-3.D4. This protocol resulted in the inclusion of a new *Xba*I site (coding for serine and arginine [SR]; Fig. 1) in lieu of the deleted sequences.

For D5 (aa 526 to 538, nucleotides 1593 to 1631), forward primer 9 and back primer 13 (c1642 to c1632 and c1592 to c1575) were used with pAcYM1.17-3 to make a PCR product with the required deleted sequences. This was recovered, cut with *Nru*I and *Dra*III, and ligated to *Nru*I- and *Dra*III-cut pAcYM1.17-3 to generate the recombinant transfer vector pAcYM1.17-3.D5.

For D6 (aa 828 to 837, nucleotides 2499 to 2528), forward primer 14 (2240 to 2258) and back primer 15 (GATCTAGA; c2498 to c2479) were used with pAcYM1.17-3 to make a PCR product corresponding to the sequence upstream of the deletion, and forward primer 16 (TATCTAGA; 2529 to 2550) and back primer 17 were used to prepare the downstream sequence. Back primer 17 corresponds to a sequence in the plasmid downstream of the VP3 coding sequence and also downstream of a *Sna*BI site. The products were recovered, digested with *Xba*I, ligated, and treated with *Sna*I (residues 2246 to 2251) and *Sna*BI before ligation to *Sna*I- and *Sna*BI-cut pAcYM1.17-3 to generate the recombinant transfer vector pAcYM1.17-3.D6.

Triplet amino acid deletions within the D4 region (D4a to D4c) were constructed by procedures similar to those used for D4. D4a lacked aa 500 to 502 (MLR; nucleotides 1515 to

TABLE 1. Oligonucleotides used for construction of deletion mutants

Mutant	Sequence	
	Forward sense primer	Reverse sense primer
D1	1 TAACAGCCATTGTAATGAGACGC	2 TCACCTTTTGCAGTGGCCCTGAGTCGCTTG
	3 AGGGCCACTGCAAAAGGTGAGGCAAGTGC	4 GTACACTTGTTCTGCAGCT
		PstI
D2	5 TGAGATCTATGGACAGATTATACTTG	6 ATACTCAACATCAATTGTAG
	BglII	
D3	7 AGCTGCAGAACAAGTGTAC	8 ATTCTAGATCTTACTGCCGGATCCATC
		XbaI
	9 TGTCTAGATTCACGAATTTAACAC	6
	XbaI	
		XbaI
D4	9	10 TTTCTAGAATTGTAACAATGATAAGTC
	11 ATTCTAGAAAAGATTCTGAGGCGGC	12 CAGCGATATCCAACTC
	XbaI	*EcoRV*
D5	9	13 AACACAGAGTGTACCATGTGAAAGGGCAG
		DraIII
	SnaI	*XbaI*
D6	14 AGTTCAGTATACTTATGAG	15 GATCTAGATATCAGATAAAAAATTGTGG
	16 TATCTAGATCTTTGGTGTTGATTAATCCAG	17 CAACGCACAGAATCTAGCGC
	XbaI	
D4a	9	18 ACTAACATCTCATTGTAACAATGATAAG
	19 TACAATGAGATGTTAGTTGCCGCAG	12
D4b	9	20 CCTGCGGCTCGTAACATCTCATTGTAAC
	21 TGTTACGAGCCGCAGGGAAAGATTC	12
D4c	9	22 AGAATCTTTAACTAACATTCGTAACATC
	23 ATGTTAGTTAAAGATTCTGAGGCGGC	12
D4aM	9	24 GGCAACTAACATTCGTAACTCATTGTA
	25 CTTATCATTGTTACAATGAGTTACGAATG	12
D4aL	9	26 GCGGCAACTAACATTCGCATCTCATT
	27 CATTGTTACAATGAGATGCGAATGTTA	12
D4aR	9	28 CCCTGCGGCAACTAACATTAACATCTC
	29 CATTGTTACAATGAGATGTTAATGTTAGTT	12

1523), D4b lacked aa 503 to 505 (MLV; nucleotides 1524 to 1532), and D4c lacked aa 506 to 508 (AAG; nucleotides 1533 to 1541). To prepare D4a, forward primer 9 and back primer 18 (c1531 to c1524 and c1514 to c1495) were used to make PCR products representing upstream DNA, and forward primer 19 (1506 to 1514 and 1524 to 1539) and back primer 12 were used to make the downstream DNA. The two PCR products were recovered, mixed, melted and used with forward primer 9 and back primer 12 to make a concatenated DNA. This was digested with *Nru*I and *Eco*RV and inserted into *Nru*I- and *Eco*RV-cut pAcYM1.17-3 to give the recombinant transfer vector pAcYM1.17-3.D4a. Similarly, to prepare D4b, forward primer 9 and back primer 20 (c1540 to c1533 and c1523 to c1504) were used to make the upstream PCR product, while forward primer 21 (1516-1523, 1533-1549) and back primer 12 were used to make the downstream product. After recovery, melting, annealing, and use with forward primer 9 and back primer 12, followed by digestion with *Nru*I and *Eco*RV, the products were ligated into *Nru*I- and *Eco*RV-cut pAcYM1.17-3 to yield the recombinant transfer vector pAc YM1.17-3.D4b. To prepare D4c, forward primer 9 and back primer 22 (c1550 to c1542 and c1532 to c1514) were used to make the upstream DNA, while forward primer 23 (1524 to 1532 and 1542 to 1558) and back primer 12 were used to make the downstream DNA. Again, the two products were recovered, melted, annealed and used with forward primer 9 and back primer 12 to recover the full-length DNA, which, after treatment with *Nru*I and *Eco*RV, was ligated into *Nru*I- and *Eco*RV-cut pAcYM1.17-3 to yield pAcYM1.17.3.D4c.

Single amino acid deletions within the D4a region were made to give D4aM lacking the methionine at aa 500 (nucleotides 1515 to 1517), D4aL lacking the leucine at aa 501 (nucleotides 1518 to 1520), and D4aR lacking the arginine at aa 502 (nucleotides 1521 to 1523). To prepare D4aM, forward primer 9 and back primer 24 (c1535 to c1518 and 1514 to c1516) were used to make the upstream DNA, and forward primer 25 (1495 to 1514 and 1518 to 1526) and back primer 12 were used to make the downstream DNA. These PCR products were recovered, melted, annealed, and used with forward primer 9 and back primer 12 to prepare the concatenated DNA. This was digested, as before, and ligated into the cut vector DNA to give pAcYM1.17-3.D4aM. To prepare D4aL, forward primer 9 and back primer 26 (c1537 to c1521 and c1517 to c1509) were used to make the upstream DNA, and forward primer 27 (1500 to 1517 and 1521 to 1529) and back primer 12 were used to make the downstream DNA. These products were recovered and used with forward primer 9 and back primer 12 to make the full-length PCR product, which, as before, was ligated to the cut vector DNA to give the recombinant transfer vector pAcYM1.17-3.D4aL. To prepare D4aR, forward primer 9 and back primer 28 (c1541 to c1524 and c1520 to c1512) were used to make the upstream DNA, and forward primer 29 (1500 to 1520 and 1524 to 1532) and back primer 12 were used to make the downstream DNA. The products were recovered, mixed, melted, annealed, and used to template PCR reactions with forward primer 9 and back primer 12 to prepare the full-length DNA. This was recovered, digested with the appropriate enzymes, and ligated to the cut transfer vector pAcYM1.17-3 to give the recombinant pAc YM1.17-3.D4aR. The mutant sequences in the derived recom-

binant transfer vectors were confirmed by using the dideoxynucleotide procedure and appropriate primers (26).

PCR. PCRs were performed with a Hybaid thermal reactor. DNA samples and oligonucleotide primers were initially denatured at 94°C for 30 s, annealed for 20 s, and extended at 72°C for 1 min. The samples were then subjected to 30 rounds of amplification.

Transfection and selection of recombinant viruses. *S. frugiperda* cells were transfected with a mixture of plasmid DNA (1 μg) and linearized BacPAK6 DNA (50 ng) as described by Kitts and Possee (12). Following staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), white plaques were recovered and the recombinant baculoviruses plaque purified as described previously (11).

SDS-PAGE analysis. *S. frugiperda* cells were infected with viruses at a multiplicity of infection (MOI) of 5 PFU per cell, and extracts of the cells were recovered at 72 h postinfection (22). Protein dissociation buffer (10% β-mercaptoethanol, 10% sodium dodecyl sulfate [SDS], 25% glycerol, 10 mM Tris-HCl [pH 6.8], 0.02% bromophenol blue) was added to each cell extract or CLP preparation, and the mixtures were heated to 100°C for 5 min. Proteins were resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE) and stained with Kenacid blue (22).

Immunoblotting analysis. Proteins resolved by SDS-PAGE were electroblotted to an Immobilon membrane (Millipore International). The membrane was incubated for 1 h at 37°C in blocking buffer (5% skimmed milk–0.05% Tween 20 in phosphate-buffered saline [PBS]). Rabbit serum containing antibodies to BTV-10 VP6 (1:1,000 dilution in washing buffer [0.05% Tween 20 in PBS]) was added, and the membrane was incubated overnight at room temperature. After four 8-min washes in washing buffer, bound antibody was detected by peroxidase-conjugated anti-rabbit antibodies (Sigma Chemical Co., St. Louis, Mo.). The ECL (enhanced chemiluminescence) system (GIBCO, BPL International) was used to detect attached conjugate.

Purification of particles. *S. frugiperda* cells were infected with each recombinant baculovirus at an MOI of 5 PFU per cell. Cells were harvested 48 h postinfection, washed with PBS, and lysed at 4°C in TNN buffer (50 mM Tris-HC [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40). Cell debris was removed by low-speed centrifugation. The particles in the extracts were then purified by banding on a discontinuous sucrose gradient (30 to 66% [wt/vol] in 0.2 M Tris-HC1 [pH 8.0]) after centrifugation at 85,000 × *g* for 2.5 h. Particles were analyzed by SDS-PAGE and by electron microscopy.

RESULTS

Analyses of the conserved domains of BTV VP3 that may be essential for core assembly. The BTV 110-kDa inner capsid protein, VP3, has a low content of charged amino acids and a high content of hydrophobic amino acids (24). VP3 sequences are highly conserved among different BTV serotypes (10, 25). VP3 has a variety of functions. It is arranged as pentamers and forms the basis for the deposition of VP7 and the formation of virus cores (7). Also, VP3 interacts with the minor proteins, VP1, VP4, and VP6 (15, 18). To maintain these functions, it is reasonable to conclude that there must be structures and sequences in VP3 that have been conserved between BTV, epizootic hemorrhagic disease virus, and African horse sickness virus. To investigate whether there are specific amino acids within the conserved regions that are important for the function(s) of VP3, the formation of CLPs and the inclusion of the minor proteins into CLPs were investigated. Six domains of VP3 largely consisting of conserved amino acids were targeted for deletion mutagenesis (Fig. 1) to determine whether mutations within these domains would abrogate the ability of VP3 to form CLPs. Deletion mutagenesis was chosen as a method for screening for important sequences in VP3 on the rationale that if particular amino acids within these sequences were required, then their removal would have a profound effect on CLP formation. The longest sequence deleted consisted of 15 aa (D3; 14 aa if one includes the substituted serine) (Fig. 1; see also Materials and Methods). Longer sequences were not investigated on the premise that large deletions would probably affect the overall structure of the protein and the function of VP3 in a nonspecific manner. Also, less specific changes would be difficult to identify through subsequent analyses involving point mutations.

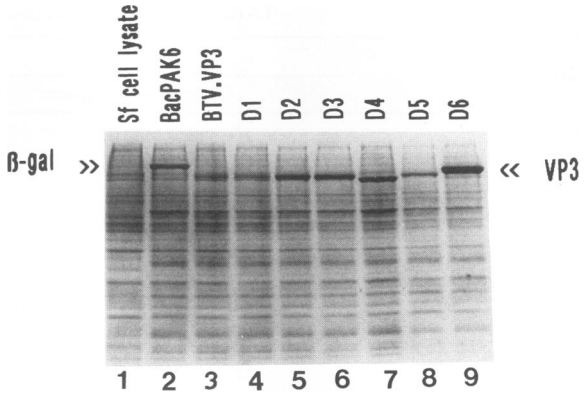
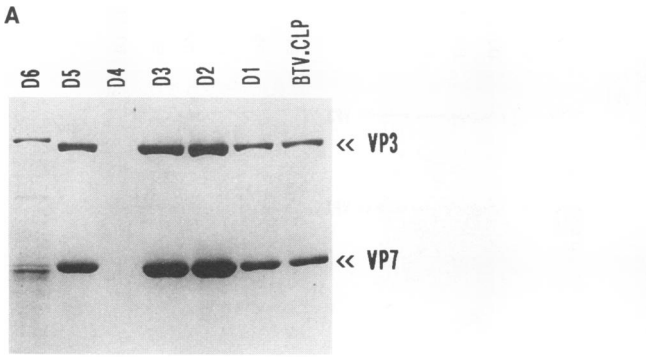
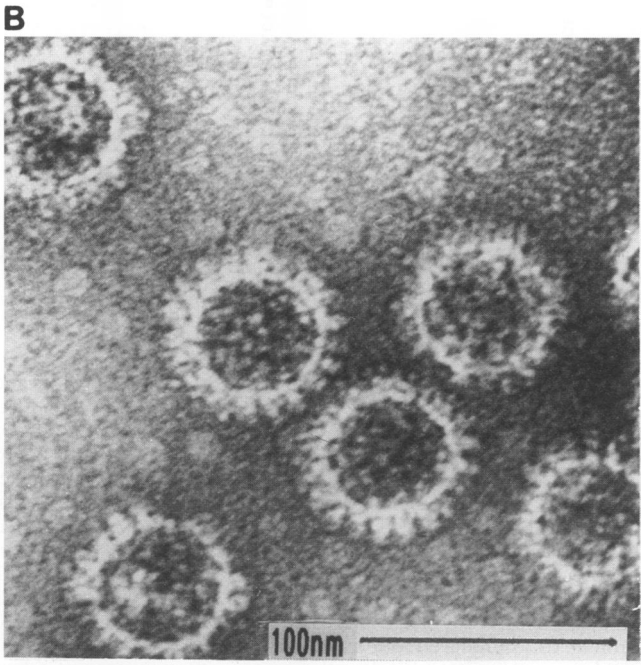


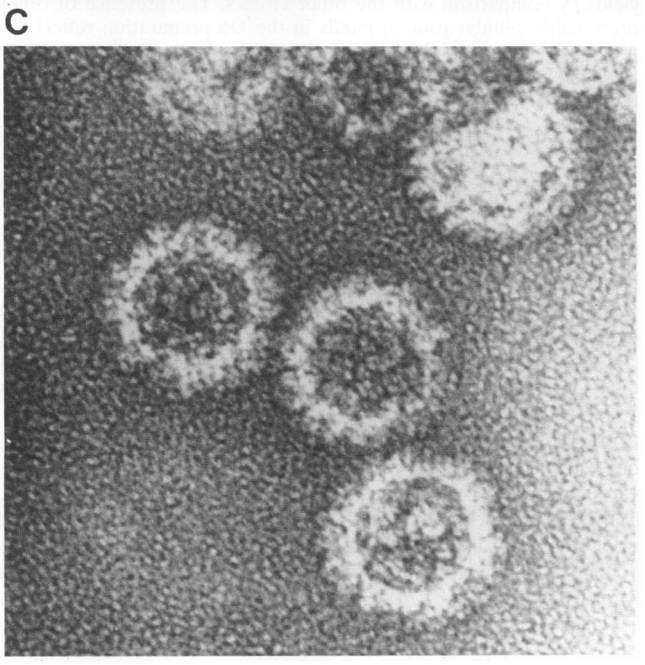
FIG. 2. Expression of the VP3 deletion mutants in insect cells. *S. frugiperda* cells were infected with each recombinant baculovirus at an MOI of 5 PFU per cell, and the cells were harvested at 3 days postinfection. After extraction in protein dissociation buffer, proteins were resolved by SDS-PAGE and stained with Kenacid blue (see Materials and Methods). Uninfected cells (lane 1) and cells infected with BacPAK6 expressing β-galactosidase (β-gal) (lane 2), served as controls. Lanes 4 to 9 contain extracts of cells infected with the baculovirus recombinant expressing an unmodified VP3 (BTV.VP3) and extracts of cells expressing the mutant VP3 species D1 to D6. The positions of the VP3 proteins and β-galactosidase are indicated.

Expression of mutant VP3 proteins and the formation of CLPs. To construct each deletion mutant, advantage was taken of unique restriction sites in the VP3 cDNA or in upstream or downstream plasmid sequences (Materials and Methods). All of the derived recombinant viruses expressed deletion forms of VP3 protein in insect cells (Fig. 2, lanes 4 to 9). Compared with uninfected cell lysates (Fig. 2, lane 1) or lysates of infections initiated with BacPAK6, which synthesizes β-galactosidase (12) (Fig. 2, lane 2), the mutant viruses made VP3 proteins in amounts and sizes essentially similar to those of unmodified BTV-17 VP3 (Fig. 2, lane 3). The expressed D6 VP3 protein was the only species that consistently migrated slightly more slowly than the unmodified VP3 or the other modified species (see other figures). The D4 VP3 protein migrated slightly faster than the other species. The reasons for these differences are not known but may reflect charge differences due to changes in the bound cation (SDS).

To determine whether the VP3 deletion mutants retained the ability to form CLPs when coexpressed with AcBTV10.7 to provide the required VP7 protein of BTV-10 (21), *S. frugiperda* cells were infected with AcBTV10.7 (21) and each of the baculoviruses that made a mutant VP3 protein (D1 to D6), using an MOI of 5 PFU per cell. A coinfection of AcBTV10-7







and AcBTV17-3, which made the unmodified VP3 protein of BTV-17, served as a control. Infected cells were lysed 3 days postinfection, and CLPs were purified in sucrose gradients as described in Materials and Methods. CLPs were observed both visually in the gradients, by electron microscopy, and by SDS-PAGE analyses of recovered materials for all coinfections except that involving D4. Shown in Fig. 3A are the protein profiles of the materials recovered from the gradients. Electron microscopic analyses confirmed the formation of CLPs (Fig. 3B and C). From these data, it was concluded that mutants D1, D2, D3, D5, and D6 produced VP3 proteins that were competent to make CLPs despite the lack of the indicated sequences, whereas mutant D4 was not. With similar experimental conditions, lower yields of CLPs were routinely obtained with the D6 construct by comparison with the other constructs that made particles. This finding may indicate that the deletion in D6 affected in some manner that affected the stability or efficiency of formation of the particles. This question was not investigated further.

The failure of D4 to make CLPs was possibly due to the presence of the additional serine and arginine at the deletion site. These amino acids were present because of the way the mutant was constructed and the presence of an inserted *Xba*I site (see Materials and Methods).

Do CLPs formed by VP7 and VP3 deletion mutants D1, D2, D3, D5, and D6 retain the ability to encapsidate the minor core proteins? It has been demonstrated previously that in insect cells, BTV CLPs formed by VP3 and VP7 proteins can incorporate each or all of the three minor proteins (VP1, VP4, and VP6) when coexpressed in the appropriate recombinant baculoviruses (15, 18). Further, it has been shown that the interactions of the minor proteins involve VP3, since removal of VP7 from CLPs leaves subcore particles composed of VP3 and the minor proteins (18). In view of the multifunctional roles of VP3, it was of interest to determine whether any of the deleted regions of VP3 were responsible for interacting with the three minor proteins. *S. frugiperda* cells were coinfected with AcBTV10.7 and D1, D2, D3, D3, or D5 and either AcBTV10-1 (for VP1), AcBTV10.4 (for VP4), or AcBTV10.6 (for VP6). After incubation for 3 days, extracts were prepared, CLPs were isolated as described in Materials and Methods, and their protein contents were analyzed by SDS-PAGE. Figure 4 shows the encapsidation of BTV minor proteins VP1 (Fig. 4A) or VP4 (Fig. 4B) in CLPs that are formed by VP7 and each of the mutant VP3 constructs tested. Despite the fact that the minor proteins were incorporated to only low levels, VP1 and VP4 were observed, albeit as faint bands, in the stained preparations of the CLPs made by each VP3 construct. Western blot (immunoblot) analyses confirmed these results (data not shown). The presence of VP6 could be detected only

FIG. 3. Protein analyses of purified CLPs. *S. frugiperda* cells were coinfected with AcBTV10-7 (to synthesize BTV-10 VP7) and either AcBTV17-3 (to synthesize unmodified BTV-17 VP3) or the individual recombinant baculoviruses expressing the VP3 deletion mutants D1 to D6. Cells were harvested 3 days postinfection, and the expressed particles were recovered and purified on discontinuous sucrose gradients (5). (A) Proteins samples were resolved by SDS-PAGE and stained with Kenacid blue. CLPs made by AcBTV10-7 and AcBTV17-3 are shown in lane BTV.CLP; those made by the coinfections involving mutants D1 to D6 are shown in the other lanes. The positions of the VP3 and VP7 proteins are indicated. (B and C) Examples of CLPs made with unmodified VP3, (B) and with mutant D2 (C) as observed by electron microscopy. CLP preparations representing the other derived products were similar (data not shown).

by Western blot analyses (Fig. 4C); stained preparations failed to identify VP6 even though the protein was made in abundance in infected cells (25). As before, constructs made by using D6 gave only low yields of CLPs; nevertheless and despite the presence of contaminating bands of protein that were probably of cellular origin, VP3, VP7, and each of the minor proteins were observed to be present. In summary, the results indicated that CLPs made in the presence of deletion mutant D1, D2, D3, D5, or D6 were able to incorporate the minor BTV proteins.

Which amino acids in D4 are required for core assembly? The initial approach undertaken to determine which residues in D4 were required for CLP formation involved the deletion of amino acid triplets in the D4 region. These deletions represented aa 500 to 502 (MLR; recombinant D4a), 503 to 505 (MLV; D4b), and 506 to 508 (AAG; D4c). Baculovirus expression vectors were made by using the appropriately modified transfer vectors (Materials and Methods). When used to express VP3, all three viruses made VP3 protein, as judged by SDS-PAGE analyses (data not shown; see Fig. 2). CLPs were prepared by coexpression of the mutant viruses with AcBTV10-7, with coexpression of AcBTV17-3 and AcBTV10-7 serving as a control. In addition to the control, only D4b and D4c yielded CLPs. This was confirmed by SDS-PAGE analyses (Fig. 5) and by electron microscopy (data not shown). The results indicated that one or more of the three amino acid residues M, L, and R (VP3 residues 500 to 502, respectively) are important for CLP assembly.

To identify which amino acids are essential for the formation of CLPs, we prepared (see Materials and Methods) three additional mutant VP3 expression vectors lacking either M (amino acid 500; recombinant D4aM), L (amino acid 501; D4aL), or R (amino acid 502; D4aR). Each recombinant was used to infect *S. frugiperda* cells, and the expression of VP3 was verified (data not shown). *S. frugiperda* cells were coinfected with AcBTV10-7 and either D4aM, D4aL, D4aR, or AcBTV17-3, and cell extracts were examined for the presence of CLPs. Apart from the control, only mutant D4aL yielded CLPs when coexpressed with VP7 (Fig. 6). From these results, it was concluded that both the VP3 M at residue 500 and the R at residue 502 were essential for CLP formation.

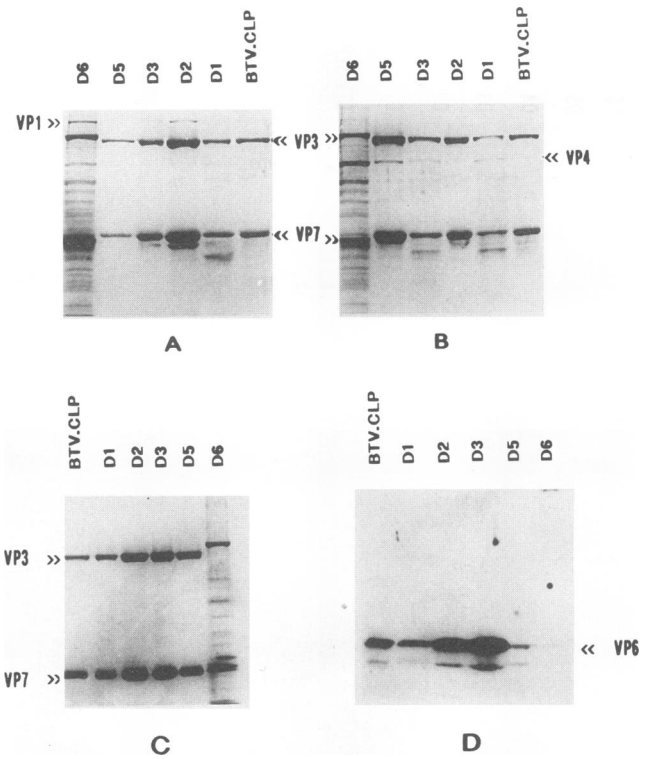


FIG. 4. Incorporation of the minor core proteins VP1, VP4, and VP6 in CLPs. *S. frugiperda* cells were coinfected at an MOI of 5 PFU of each virus per cell with AcBTV10-7 and either D1, D2, D3, D5, or D6 in the presence of AcBTV10.1 (A), AcBTV10.4 (B), or AcBTV10-6 (C and D). The cells were harvested at 3 days postinfection, and CLPs were purified as described in Materials and Methods. The particles were recovered, analyzed by SDS-PAGE, and stained with Kenacid blue (A to C) or reacted with anti-VP6 serum in Western analyses (D). The positions of the VP1, VP3, VP4, VP6, and VP7 proteins are indicated. The lanes labeled BTV.CLP represent CLPs made from AcBTV17-3 and AcBTV10-7 and the corresponding minor protein. CLPs produced with VP3 mutant D6 were recovered in lower yields by comparison with the other viruses. The presence of other, presumably cellular protein bands in the D6 preparation reflect this problem. Apart from D6, minor bands in the other CLP preparations probably are contaminant cellular proteins or degradation products of the expressed proteins.

DISCUSSION

The sequences of the five core proteins of BTV are highly conserved between the virus serotypes that have been analyzed to date. In addition, among the gnat-transmitted orbiviruses (BTV, epizootic hemorrhagic disease virus, and African horse sickness virus), the VP3 proteins have been shown to have a high degree of sequence identity. These data indicate that there are conserved functions in VP3 probably involving conserved amino acids and sequences of amino acids. Using the formation of CLPs as an assay, it has been shown that VP3 of BTV can interact with the VP7 of epizootic hemorrhagic disease virus when coexpressed, and vice versa (reference 15 unpublished data).

The BTV VP3 subcore has T=1 symmetry. Cryoelectron microscopy analyses (7) indicate that VP3 is composed of 12 closely bonded pentamers located at each of the fivefold axes of the subcore. Whether these pentamers are composed only of monomers of VP3 or some polymer of VP3 (e.g., VP3 dimers) is not known. VP3 pentamers appear to have two protrusions which link them to a neighboring VP3 pentamer. The 260 surface VP7 trimers of the core form a T=13 lattice on top of the VP3 subcore. This gives a 13:1 ratio between the 780 molecules of VP7 and the 60 pentameric components of VP3. How VP3 and VP7 interact is not yet known. Expression of VP3 in the baculovirus system has failed to identify the formation of subcores, unlike the corresponding protein of rotaviruses (13). Also, because of insolubility problems, no evidence for pentamers of VP3 has been obtained for the singly expressed VP3 (unpublished observations). However, coexpression of VP7 and VP3 produces CLPs. On dialysis, CLPs yield subcores (18), which are less stable than cores. Thus, it appears that interaction with VP7 leads to a stabilization of VP3 in the subcores. To identify interacting sequences between the two main proteins of the BTV core in this study, we examined a series of mutant VP3 proteins of BTV for the ability to form CLPs with BTV VP7 and to incorporate the minor structural proteins of the virus.

Two VP3 amino acids, residues 500 (M) and 502 (R), were found to be critical for CLP formation. These were identified by a series of deletion mutants involving progressively smaller deletions. Surprisingly, for five of the conserved domains examined (D1, D2, D3, D5, and D6), little or no effect was

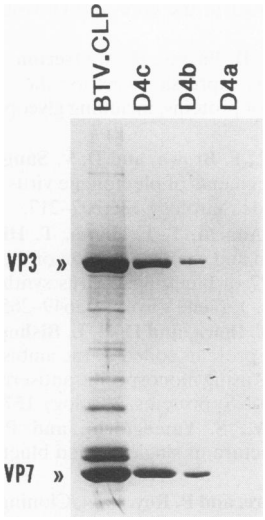


FIG. 5. Formation of CLPs with use of VP3 mutants D4a, D4b, and D4c. *S. frugiperda* cells were infected at an MOI of 5 PFU per cell with AcBTV10-7 and either AcBTV17-3 (BTV.CLP), D4a, D4b, or D4c. The CLPs were purified and analyzed by SDS-PAGE as described in Materials and Methods. The positions of VP3 and VP7 in the stained gels are indicated. Minor protein bands probably represent cellular contaminants and, possibly, degradation products of the expressed proteins.

observed with the deletions, even for deletions involving conserved sequences that were up to 15 aa long. An exception to this observation may be the D6 domain, as indicated by the low yields of CLPs that were obtained. All of the VP3 mutants which made CLPs also incorporated the three minor proteins. These data do not exclude the possibility that other conserved functions (e.g., ability to interact with RNA) might be affected by the mutations; however, until assays are established to investigate such alternative interactions, this cannot be determined.

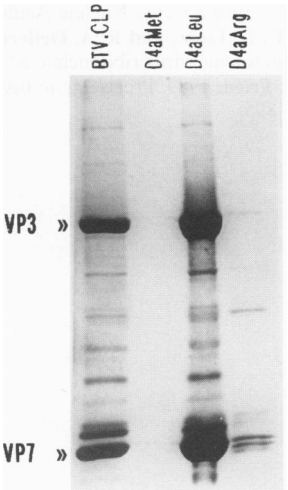


FIG. 6. Formation of CLPs with use of VP3 mutants D4aM, D4aL, and D4aR. *S. frugiperda* cells were infected at an MOI of 5 PFU per cell with AcBTV10-7 and either AcBTV17-3 (BTV.CLP), D4aM, D4aL, or D4aR. The CLPs were purified and analyzed by SDS-PAGE as described in Materials and Methods. The positions of VP3 and VP7 in the stained gels are indicated. Minor protein bands probably represent cellular contaminants and, possibly, degradation products of the expressed proteins.

Discounting the possibility that the VP3 proteins were folded incorrectly as a result of the particular substitutions that were made, there are two possible reasons that can be considered for the failure of the mutant VP3 proteins to form CLPs when aa 500 and 502 were altered. One is an effect on VP3-VP3 interactions and the formation of stable pentamers or on interactions between pentamers in the subcore structure. The other is an effect on the interactions between VP3 and VP7. Neither of these possibilities can be investigated with the present assay, since subcores of VP3 do not form when VP3 is expressed alone. In view of their proximity, it is probable that the two amino acids form a site of interaction with another site on either the same VP3 molecule, another VP3, or VP7. Whether there are other amino acids in the region of residues 500 to 508 that are involved can be investigated by similar deletion and site-directed mutagenesis of sequences upstream of residue 500 or downstream of residue 508, although it should be noted that deletion of residues 526 to 538 (D5) had no effect on CLP formation.

In view of the triplet deletion analyses, it is probable that both methionine and arginine are required amino acids for a function of VP3. While the importance of arginine in various functions and intermolecular interactions has been well documented in other systems (4, 16), there is less evidence for the specific participation of methionine. No investigation has been conducted to determine whether substitutions other than introducing leucines at residues 500 and 502 allow CLP formation. Leucine, although a neutral and hydrophobic amino acid, may be nonpermissive because of its side chain. Other amino acids lacking large side chains (e.g., glycine or alanine) may be investigated to confirm the requirement for methionine. Similarly, it would be of interest to determine whether amino acids of similar charge (e.g., lysine at 502 instead of arginine) would be permissive. Whether the positions of methionine and arginine are critical has yet to be determined (e.g., by introducing amino acids in between, by reversing their locations, etc.). Until VP3 is crystallized and a three-dimensional structure is obtained as for VP7 (reference 1 and unpublished data), it will not be possible to determine why these particular VP3 sequences are critical.

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