JOURNAL OF VIROLOGY, Dec. 1990, p. 5695–5700
0022-538X/90/125695-06$02.00/0

Vol. 64, No. 12

Assembly of Double-Shelled, Viruslike Particles of Bluetongue Virus by the Simultaneous Expression of Four Structural Proteins

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Received 29 June 1990/Accepted 24 August 1990

Bluetongue is a disease of ruminants. The etiologic agent is bluetongue virus (BTV), a gnat-transmitted member of the *Orbivirus* genus of the *Reoviridae*. The virus has a genome of 10 double-stranded RNA species L1 to L3, M4 to M6, S7 to S10). The L2 and M5 genes of BTV which encode the outer capsid proteins VP2 and VP5, respectively, were inserted into a recombinant baculovirus downstream of duplicated copies of the baculovirus polyhedrin promoter. Insect cells coinfected with this virus plus a recombinant baculovirus expressing the two major core proteins VP3 and VP7 of BTV (T. J. French and P. Roy, J. Virol. 64:1530–1536, 1990) synthesized noninfectious, double-shelled, viruslike particles. When purified, these particles were found to have the same size and appearance as authentic BTV virions and exhibited high levels of hemagglutination activity. Antibodies raised to the expressed particles contained high titers of neutralizing activity against the homologous BTV serotype. The assembly of these bluetongue viruslike particles after the simultaneous expression of four separate proteins is indicative of the potential of this technology for the production of a new generation of viral vaccines and for the study of complex, multiprotein structures.

Gene cloning and expression have proved to be powerful tools for assessing the structural and/or functional roles of individual proteins. The natural progression for future studies will be the examination of multiprotein structures such as viruses or enzyme complexes. The assembly of viruslike particles (VLPs) after the synthesis of their component proteins is an area which presents a number of opportunities. For example, the stages of viral assembly, the contributions of individual components to that process, and the sites and nature of viral protein interactions can be studied. An increased understanding of viral morphogenesis may aid the development of antiviral agents which specifically interfere with the assembly process. Intact VLPs could also prove useful as vaccines if epitopes are presented in authentic conformations.

To date, only single-shelled VLPs or cores have been assembled after the expression of one (11, 16) or two (3) viral genes. For viruses with structures derived from the translation products of more than one mRNA, coexpression of different genes is required if essentially complete, albeit empty, particles are to be assembled. With such an objective in mind, baculovirus multiple expression vectors have been developed (2). One such vector, pAcVC3, contains duplicated copies of the polyhedrin transcriptional machinery from *Autographa californica* nuclear polyhedrosis virus (AcNPV). This enables a recombinant baculovirus to be constructed which will express two foreign polypeptides simultaneously in *Spodoptera frugiperda* insect cells (3, 16).

Bluetongue virus (BTV) is a member of the *Orbivirus* genus in the family *Reoviridae*. It is an arthropod-borne virus that causes disease in sheep and cattle. It has a genome consisting of 10 segments of double-stranded RNA (L1 to L3, M4 to M6, S7 to S10) that are located in a core particle consisting of two major proteins (VP3 and VP7) and three minor proteins (VP1, VP4, and VP6). In intact virus, an outer capsid of two major proteins (VP2 and VP5) surrounds the core. Three nonstructural proteins are synthesized in BTV-infected cells. Their functions are unknown.

We have previously described the construction of a dual recombinant baculovirus that expresses both VP3 and VP7 and shown that empty corelike particles (CLPs) are assembled in insect cells infected with this recombinant virus (3). When used to immunize animals, these single-shelled CLPs do not protect against subsequent BTV infection because the epitopes that elicit neutralizing antibodies are present on the outer capsid protein VP2 (4–6). Since a recombinant baculovirus expressing VP2 has been constructed (6), we initially undertook coinfection experiments with the VP3 and VP7 dual vector and the VP2 vector in an attempt to determine whether VP2 became associated with the CLPs. No VP2 was detected on particles purified from insect cells expressing VP2, VP3, and VP7 (data not shown). Similar results were obtained in a parallel study with the VP3 and VP7 vector together with a recombinant baculovirus expressing VP5 (9). Since neither of the capsid proteins attached individually to the CLPs, a dual recombinant baculovirus was constructed which would express both VP2 and VP5 simultaneously. Coinfection experiments with both dual recombinant baculoviruses then enabled us to examine the interactions of all four major BTV structural proteins when simultaneously expressed in insect cells.

MATERIALS AND METHODS

DNA manipulation and construction of dual transfer vector. Plasmid DNA was manipulated by following the procedures described by Maniatis and associates (8). Restriction enzymes, T4 DNA ligase, and the Klenow large fragment of DNA polymerase were purchased from Amersham International plc (Buckinghamshire, United Kingdom). Calf intestinal alkaline phosphatase was obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany).

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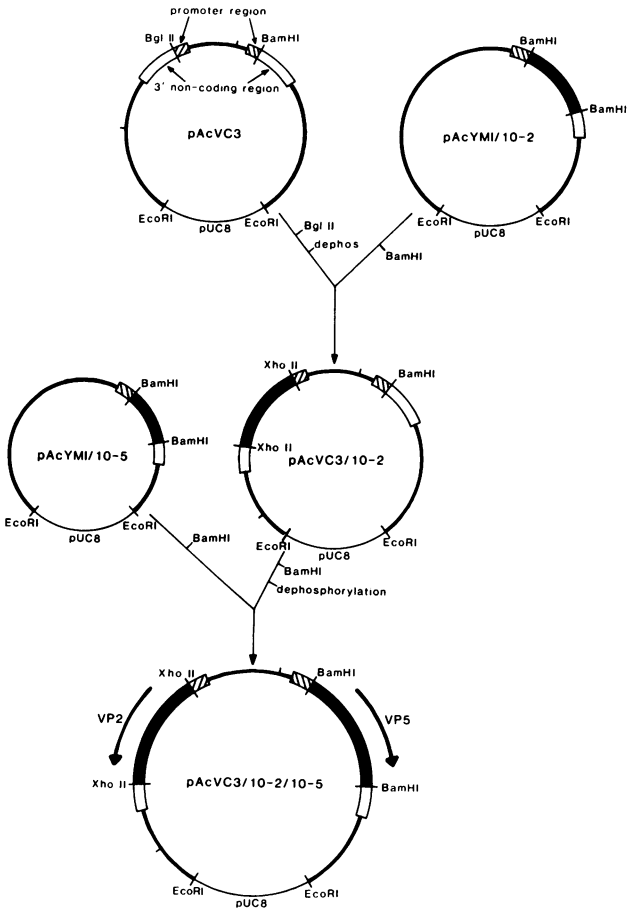

FIG. 1. Construction of the baculovirus expression transfer vector containing the L2 and M5 genes of BTV serotype 10. The cloning, genetic manipulations, and individual expression of these genes have previously been described (6, 9, 12, 13). The L2 and M5 genes were excised from their single baculovirus expression transfer vector (pAcYM1) and ligated into the *Bgl*II and *Bam*HI sites, respectively, of the multiple expression vector pAcVC3.

The manipulations for the construction of the VP2-VP5 recombinant plasmid are shown in Fig. 1. They involved excision of the L2 and M5 genes from their pAcYM1 single expression transfer vector and insertion into the *Bam*HI and *Bgl*II sites, respectively, of the multiple expression vector pAcVC3.

Monolayers of *S. frugiperda* cells were cotransfected with this dual transfer vector DNA (pAcVC3/BTV-10-2/BTV-10-5) and wild-type AcNPV DNA by the standard method of calcium chloride precipitation (15). Recombinant baculoviruses generated by homologous recombination were selected by virtue of their polyhedrin-negative phenotype and plaque purified.

SDS-PAGE analysis and purification of expressed particles. *S. frugiperda* cells were infected at a multiplicity of 10^5 PFU per cell with either the recombinant baculoviruses or wild-type AcNPV or were mock infected. Cells were harvested at 48 h postinfection, washed with phosphate-buffered saline, and lysed at 4°C in 50 mM Tris hydrochloride (pH 8.0)–150 mM NaCl–0.5% Nonidet P-40. Protein dissociation buffer (10% β-mercaptoethanol, 10% sodium dodecyl sulfate [SDS], 25% glycerol, 10 mM Tris hydrochloride [pH 6.8], 0.02% bromophenol blue) was added to each sample, and the mixture was heated to 100°C for 5 min. Proteins were resolved by SDS-10% polyacrylamide gel electrophoresis (PAGE) (17) and stained with Coomassie brilliant blue.

The expressed VLPs were purified by lysing the cells as described above and banding on a discontinuous sucrose gradient (30%:50% [wt/vol] in 0.2 M Tris hydrochloride [pH 8.0]) after centrifugation at 85,000 × *g* for 3 h. The particles were analyzed by SDS-PAGE (as above) or were examined by electron microscopy. BTV particles were purified from monolayers of BHK-21 cells infected with BTV serotype 10 as described previously (10).

Western immunoblot analysis. Proteins were separated by SDS-PAGE and electroblotted onto Immobilon (Millipore Corp., Bedford, Mass.) with a Sartorius semi-dry electroblotter. The filter was soaked overnight at 4°C in blocking buffer (5% skim milk, 0.05% Tween 20 in phosphate-buffered saline) and then rabbit anti-BTV-10 serum (1:1,000 dilution in fresh blocking buffer) was added and the filter was gently agitated for 90 min at room temperature. After being washed for 1 h (0.05% Tween 20 in phosphate-buffered saline), the filter was returned to blocking buffer containing a 1:1,000 dilution of goat anti-rabbit immunoglobulin G (Sigma) and gently agitated at room temperature for 90 min. The filter was then washed again and placed in substrate (β-naphthyl phosphate disodium, fast blue BB salt, $MgSO_4$) for 5 min before rinsing in water and drying. Since VP2 comigrates with VP3, its presence in the purified double-shelled VLPs and authentic BTV virions was confirmed by repeating the above protocol but reacting the filter with antiserum raised to expressed VP2 (6).

Electron microscopy. Purified BTV-10 virions and expressed double-shelled VLPs were absorbed 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 hydrochloride (pH 8.0), the particles were stained for 20 s on droplets of 2% uranyl acetate. All grids were examined in a JEOL electron microscope.

Preparation of antibodies against VLPs, CLPs, and baculovirus-expressed BTV proteins. The VLPs and CLPs were purified as described above, and approximately 75 μg of each material was injected intraperitoneally into guinea pigs at days 0, 7, 14, and 21. For anti-VP2 serum, infected *S. frugiperda* cell lysates containing VP2 protein were prepared as described previously (9). Guinea pigs were similarly injected four times, each inoculum containing approximately 50 μg of VP2 protein. Serum samples were collected at day 31 by cardiac puncture from anesthetized guinea pigs. Preparation of rabbit antisera to VP2 or VP7 and mouse antisera to VP3 or VP5 has been described previously (6, 9).

Neutralization and hemagglutination activity of antisera raised to expressed particles. Guinea pig antisera raised to expressed CLPs or VLPs or antisera raised to other BTV proteins were assayed for their neutralizing activity against BTV-10 in a plaque reduction test. A total of 200 PFU of BTV-10 were incubated with serial dilutions of antisera (in phosphate-buffered saline) at 37°C for 1 h before inoculating monolayers of BHK cells. After virus absorption at room temperature for 1 h, the inoculum was discarded and replaced with medium containing 0.8% agarose. After incubation for 3 days at 37°C, the plaques were visualized by applying a neutral red stain overlay.

Hemagglutination tests were performed in microtiter plates as described by Van Der Walt (18). Serial dilutions of the purified particles were produced with TSAG buffer (10 mM Tris chloride [pH 9.0], 140 mM NaCl, 1 mM $CaCl_2$, 0.2% [wt/vol] bovine serum albumin fraction V). Erythro-

cytes from rabbits were washed with Alsevier solution and then diluted with TSAG to produce a 0.25% suspension. A 50-μl portion of this suspension was added to each serial dilution and left overnight at 4°C. One HA unit is defined as the highest dilution at which hemagglutination occurs. The antisera to be tested as inhibitors were diluted serially with TSAG. Four HA units of the purified particles in a 25-μl total volume of TSAG were then added to each well and left overnight at 4°C. Erythrocytes (50 μl of a 0.25% suspension) were then added, and the mixture was left overnight at 4°C.

RESULTS

Construction of dual recombinant baculovirus. cDNA copies representing the complete coding sequences of the BTV L2 and M5 genes were manipulated for expression in the baculovirus system as described in Materials and Methods (Fig. 1). Recombinant baculoviruses were prepared by the established procedure of cotransfecting *S. frugiperda* insect cells with the recombinant plasmid DNA and wild-type AcNPV DNA (15). Progeny viruses were titrated by using confluent monolayers of *S. frugiperda* cells, and putative recombinants were selected on the basis of their polyhedrin-negative phenotype (ca. 0.1% frequency). After successive rounds of plaque purification, a high-titered viral stock was prepared.

Coexpression of VP2 and VP5 in *S. frugiperda* cells. *S. frugiperda* cells infected with the recombinant baculovirus synthesized two unique protein species in place of the 29-kilodalton polyhedrin protein seen in wild-type AcNPV-infected cells (Fig. 2A). The sizes of the expressed proteins agree with those expected for VP2 and VP5 calculated from their amino acid compositions (i.e., 111.1 and 59.1 kilodaltons, respectively [12, 13]). Since the levels of expression were below that which could be determined by staining, confirmation that the expressed proteins represented authentic BTV proteins was provided by Western immunoblot analyses with antiserum raised to BTV-10 virus particles (Fig. 2B). This antiserum reacted well with the VP2 and VP5 species in a cell lysate of *S. frugiperda* cells infected with the dual recombinant baculovirus. No reaction was detected with mock-infected or wild-type AcNPV-infected cells. BTV-10 virus particles were included as a control, from which it can be seen that the BTV-10 antisera also contained antibodies to the minor core protein VP6 (but not to VP1 or VP4).

Although the outer capsid of BTV is composed solely of VP2 and VP5, no capsidlike structures were detected in insect cells infected with the VP2-VP5 recombinant baculovirus. The absence of VP2-VP5 complexes in the infected insect cells was further confirmed by examination of immunoprecipitates of the infected cells with monospecific antisera to VP2 or VP5. Each antiserum precipitated only one protein, either VP2 or VP5 (data not shown), indicating that the VP2 and VP5 proteins were not present as complexes in the insect cells.

To assess the interaction of VP2 and VP5 with the BTV CLPs, insect cells were coinfected with both dual recombinant baculoviruses (to coexpress VP2, VP3, VP5, and VP7). The cells were harvested at 48 h postinfection and lysed with the nonionic detergent Nonidet P-40, and the released particles were purified to homogeneity by centrifugation on discontinuous sucrose gradients. When examined under the electron microscope, empty double-shelled particles were observed consisting of a core surrounded by a thick outer capsid (Fig. 3A, large arrows). The diameters of the largest

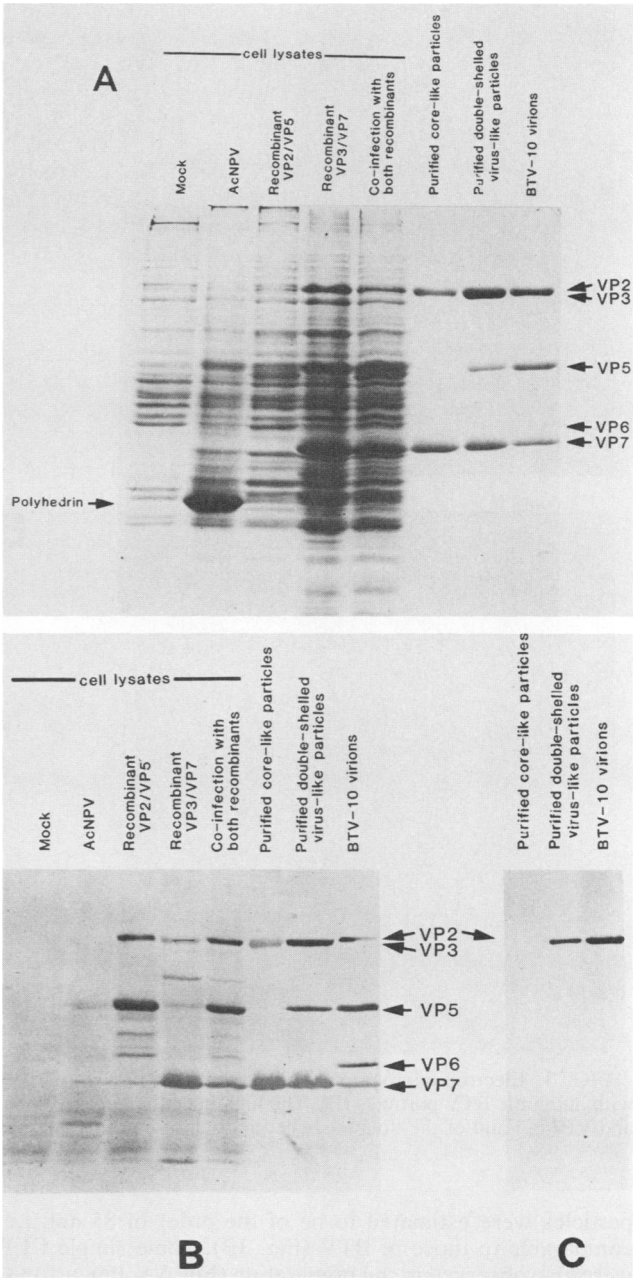

FIG. 2. Expression of the four major BTV structural proteins VP2, VP3, VP5, and VP7 in insect cells by recombinant baculoviruses and confirmation of their authenticity by Western immunoblot analysis. *S. frugiperda* cells were infected at a multiplicity of 5 PFU per cell with either the recombinant baculovirus expressing VP2 and VP5 or the recombinant expressing VP3 and VP7 or were coinfected with both recombinant viruses. Mock-infected and wild-type AcNPV-infected cells acted as controls. Authentic BTV virions prepared from BTV-infected BHK cells are included for comparison. Proteins were separated by SDS-PAGE and stained with Coomassie blue (A) or were electroblotted onto Immobilon membrane and reacted with rabbit BTV-10 antiserum (B). Since VP2 comigrates with VP3, its presence in the purified double-shelled VLPs and authentic BTV virions was confirmed by reactions with antiserum raised to expressed VP2 (C). Bound antibody was detected with an alkaline phosphatase conjugate by standard methods.

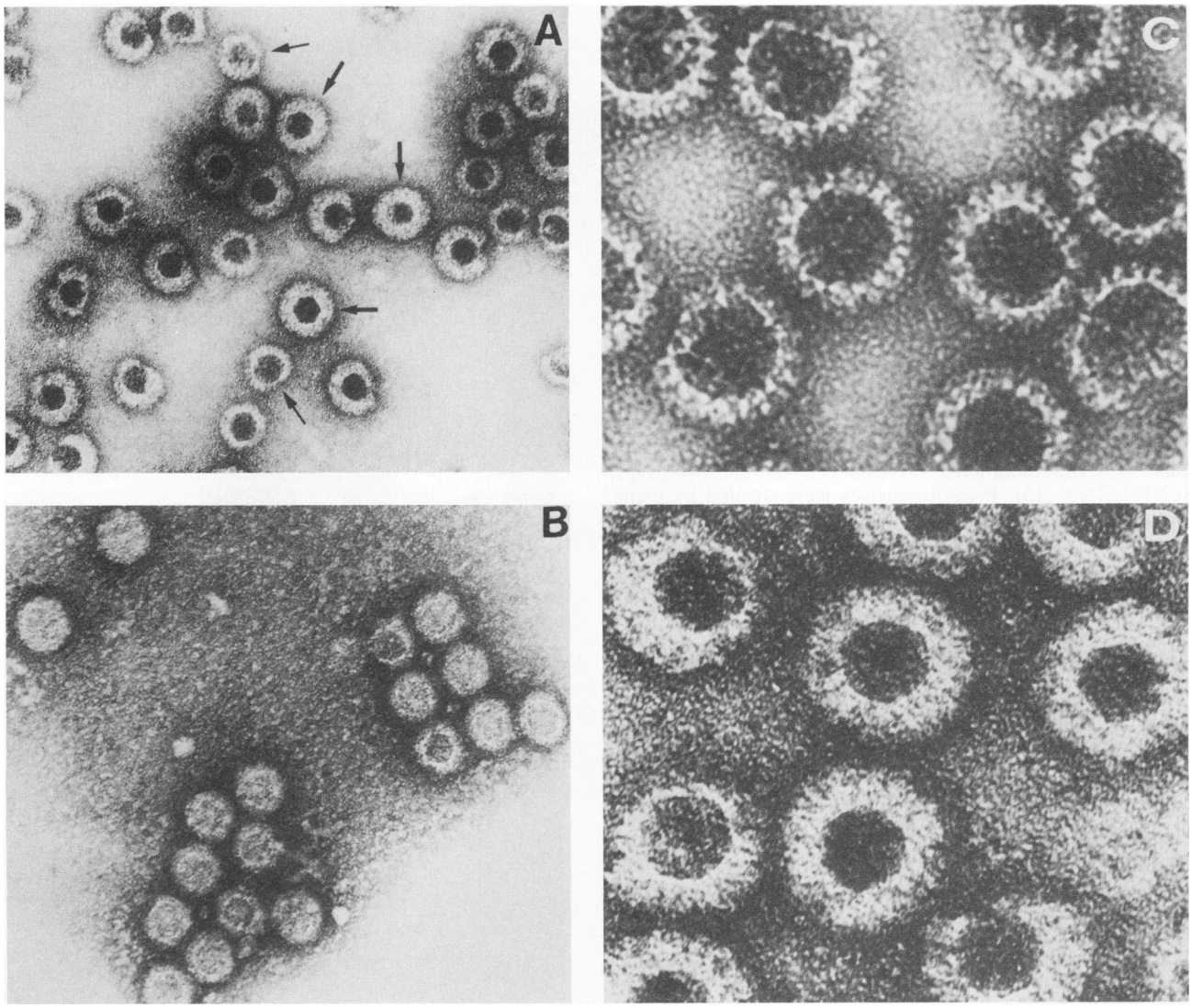

FIG. 3. Electron micrographs of baculovirus-expressed particles. Empty BTV double-shelled VLPs are shown in panel A and compared with authentic BTV particles (B). The high-magnification micrographs (×30,000) show the appearance of expressed CLPs composed of VP3 and VP7 (C) and of the double-shelled particles with VP2 and VP5 attached to VP3 and VP7 (D).

particles were estimated to be of the order of 85 nm, i.e., comparable to those of BTV (Fig. 3B). Some simple CLPs were also observed in the preparation (Fig. 3A, thin arrows). Their diameters were estimated to be of the order of 65 nm. A range of intermediate structures were also observed, apparently with various amounts of the outer capsid proteins attached. These may reflect different stages in particle assembly. Interestingly, the centers of all types of particles (CLPs, VLPs, intermediate VLPs) exhibited an icosahedral configuration. The smaller size of the central area of the VLPs is presumably due to the presence and density of the outer capsid proteins. The icosahedral configuration of the center was also apparent in several authentic BTV particles where stain had penetrated the particles. The expressed particles were analyzed by SDS-PAGE and Western immunoblot analyses and shown to contain large amounts of VP2 and VP5 (Fig. 2) in addition to VP3 and VP7. However, due to the various amounts of VP2 and VP5 proteins attached to the cores, the stoichiometries of the proteins were not determined. Phenol extraction of purified expressed particles and examination by optical density measurements or agarose gel electrophoresis failed to demonstrate the presence of nucleic acid.

Hemagglutination and immunogenicity of expressed VLPs. The immunogenicity and hemagglutinating activity of the VLPs were investigated. Guinea pig sera raised against purified CLPs and the double-shelled VLPs were tested for their ability to neutralize the infectivity of BTV-10. As expected, serum raised to the cores exhibited no neutralizing activity, while in a 50% plaque reduction test, substantial neutralization was demonstrated by the serum raised to the double-shelled VLPs at a dilution of 1:10,000. Monospecific serum raised in guinea pigs to VP2 gave titers of <640. As expected, none of the other three monospecific sera (to VP3, VP5, or VP7) raised in rabbits or mice neutralized BTV. Purified VLPs exhibited hemagglutinating titers (Table 1) as is observed with authentic virus (18). Purified CLPs did not hemagglutinate. These data agree with the demonstration

TABLE 1. Hemagglutination analysis of BTV double-shelled VLPs[a]

Substrate or serum tested	HA or HI titer	Plaque reduction neutralization titer
Substrate (20 μg)	HA titer	
Single-shelled CLPs	<2	
Double-shelled VLPs	2,048	
Serum	HI titer	
Preimmune GP	4	0
GP anti-VLP	>2,048	10,000
GP anti-CLP	<2	0
GP anti-VP2	Not done	<640
Preimmune rabbit	16	0
Rabbit anti-VP2	>1,024	>640
Rabbit anti-VP7	2	0
Preimmune mouse	4	0
Mouse anti-VP5	8	0
Mouse anti-VP3	32	0

[a] HA titers are expressed as the reciprocal of the highest serial dilution that gave complete hemagglutination. Antisera raised to the particles and baculovirus-expressed BTV proteins were used in hemagglutination inhibition (HI) and plaque reduction neutralization tests. The inhibition titers are expressed as the reciprocal of the highest serial dilution of the serum that gave complete inhibition of hemagglutination. The plaque reduction neutralization titers are expressed as the reciprocal of the serum dilution that gave a 50% reduction in plaque numbers. Guinea pig (GP), rabbit, and mouse sera were used as indicated.

that VP2 is the hemagglutinating protein of BTV (18). Monospecific serum raised to VP2 inhibited hemagglutination by the VLPs. Monospecific sera raised to the other component proteins (VP3, VP5, or VP7) had essentially no effect (Table 1). Unlike authentic BTV, the VLPs were noninfectious when assayed in mammalian cells.

DISCUSSION

BTV cores are composed of two major and three minor proteins in addition to the 10 segments of double-stranded RNA. An outer capsid of two major proteins surrounds the core. Coexpression of the two major core proteins (VP3 and VP7) in insect cells by a multiple recombinant baculovirus results in the synthesis of CLPs of the same size, appearance, and stoichiometric arrangement of VP3 to VP7 as authentic BTV core particles (3). Attempts to attach the outer capsid proteins (VP2 and VP5) individually to these CLPs by coinfecting insect cells with the VP3-VP7 dual recombinant and either a recombinant expressing VP2 or one expressing VP5 were unsuccessful. A second dual recombinant expressing both VP2 and VP5 simultaneously was therefore constructed. Unlike the two core proteins, this dual recombinant virus failed to form any capsid or VP2-VP5 complex in the insect cells. It is likely that the two proteins need the core structure before they can interact. When insect cells were coinfected with the VP2-VP5 recombinant plus the VP3-VP7 recombinant, empty double-shelled VLPs were assembled. The formation of these particles leads to several interesting conclusions regarding BTV morphogenesis. The failure of VP2 and VP5 to attach individually to the cores suggests that the interaction of these proteins occurs during the process of assembly of VLPs. As with the formation of CLPs in insect cells, the addition of the outer capsid is not dependent on the presence of the BTV nonstructural proteins (NS1, NS2, NS3), or viral double-stranded RNA, or the minor proteins VP1, VP4, and VP6.

The potential of these double-shelled VLPs as a new type of BTV vaccine (in sheep) is currently under investigation. A range of homologous and heterologous BTV serotypes exist; thus, effective protection would necessitate the use of a cocktail of particles (or chimeric particles) containing the appropriate VP2 proteins. We are currently constructing such particles by attaching VP2 and VP5 proteins representing a selection of BTV serotypes to the core particles.

ACKNOWLEDGMENTS

This research was supported by funds from Oxford Virology, ARI 89-401, and by Public Health Service grant AI26879 from the National Institutes of Health.

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