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Synthesis of Bluetongue Virus (BTV) Corelike Particles by a Recombinant Baculovirus Expressing the Two Major Structural Core Proteins of BTV

T. J. FRENCH[1] AND P. ROY[1,2]*

NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, OX1 3SR United Kingdom,[1] and Department of Environmental Health Sciences, School of Public Health, University of Alabama, Birmingham, Alabama 35294[2]*

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The L3 and M7 genes of bluetongue virus (BTV), which encode the two major core proteins of the virus (VP3 and VP7, respectively), were inserted into a baculovirus dual-expression transfer vector and a recombinant baculovirus expressing both foreign genes isolated following in vivo recombination with wild-type *Autographa californica* nuclear polyhedrosis virus DNA. *Spodoptera frugiperda* insect cells infected with the recombinant synthesized large amounts of BTV corelike particles. These particles have been shown to be similar to authentic BTV cores in terms of size, appearance, stoichiometric arrangement of VP3 to VP7 (ratio, 2:15), and the predominance of VP7 on the surface of the particles. In infected insect cells, the corelike particles were observed in paracrystalline arrays. The formation of these structures indicates that neither the BTV double-stranded viral RNA species nor the associated minor core proteins are necessary for assembly of cores in insect cells. Furthermore, the three BTV nonstructural proteins NS1, NS2, and NS3, are not required to assist or direct the formation of empty corelike particles from VP3 and VP7.

Empty, single-shelled virus structures have been assembled for a number of different viruses from single structural proteins both in vivo, e.g., hepatitis B virus (12, 16) and in vitro, e.g., rotaviruses (14). In both of these examples, the observed particles were synthesized from a single protein species. Recently, by using baculovirus vectors, empty poliovirus particles containing equimolar amounts of three different protein species (VP0, VP1, and VP3) have been synthesized (17). In the latter case, the protein coat of poliovirus was derived by processing the translation product of a polycistronic mRNA. Viral structures which contain multiple polypeptide species encoded by separate genes (mRNA species) and involving nonequimolar ratios of proteins present a more difficult and challenging objective to studies of the processes of viral morphogenesis.

Formation of complex structures and evaluation of the interactions of their protein components can be attempted in vitro, but this may introduce artifacts, depending on the experimental conditions used (14). Synthesis of proteins in eucaryotic cells by an expression vector provides an opportunity to investigate macromolecular interactions under more natural, intracellular conditions.

There are numerous reports of high-level expression of foreign proteins by recombinant baculoviruses (reviewed in reference 8), and insect cells have been demonstrated to provide an appropriate environment for the assembly of foreign proteins (1, 16, 17). Until recently, a limitation of the baculovirus expression system has been that simultaneous expression of several proteins within a single cell requires coinfection with two recombinant viruses, each containing a single foreign gene. Success by this approach depends upon achievement of efficient infection at a high multiplicity of infection for each virus (1, 11). This not only requires large amounts of high-titered virus stocks but also makes reproducibility between experiments difficult to achieve. To overcome such problems, baculovirus multiple-expression vectors, such as pAcVC3, have been constructed (3) and used to express more than one foreign gene (16). This vector has two copies of the polyhedrin promoter and transcription termination sequences. In plasmid transfer vector pAcVC3, unique enzyme restriction sites are located downstream of each copy of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) polyhedrin promoter. The sites allow two foreign genes to be placed under the control of their own copy of the polyhedrin transcriptional machinery. The promoters are arranged in opposite orientations to minimize the possibility of homologous sequence recombination and excision of either of the foreign genes (see Fig. 1).

Bluetongue virus (BTV) is an arthropod-borne virus that causes disease in sheep and cattle. It is prevalent in many areas of the world and is of economic importance to the livestock industry. The virus is a member of the *Orbivirus* genus in the family *Reoviridae* and has a genome consisting of 10 segments of double-stranded RNA located in a core particle consisting of two major proteins, VP3 and VP7 (together with three minor proteins, VP1, VP4, and VP6). An outer capsid of two major proteins, VP2 and VP5, surrounds the core. The function(s) of the core proteins has not been determined, and little is known about their interactions and stoichiometric arrangements. Appreciation of such factors is important if the morphogenesis of the virus is to be understood. Recent cryoelectron microscopy studies (B. V. Ventakaram Prasad et al., manuscript in preparation) suggest that the core of BTV consists of a nucleoprotein center surrounded by two distinct protein layers, each of which may be composed of a single polypeptide species. Immunogold analysis indicate that VP7 is the principal component of the outermost layer and is attached to a framework of VP3 (6). To determine whether particles resembling BTV cores could be synthesized from the VP3 and VP7 species, a dual-recombinant baculovirus containing the BTV L3 and M7 gene segments (which encode VP3 and

* Corresponding author.

VP7, respectively) was constructed. *Spodoptera frugiperda* cells infected with this recombinant synthesized large quantities of VP3 and VP7 which assembled to form empty BTV corelike particles. These were purified and demonstrated by electron microscopy to be essentially identical in size and appearance to authentic BTV core particles.

MATERIALS AND METHODS

DNA manipulations and construction of a dual-transfer vector. Plasmid DNA manipulations were performed by the procedures described by Maniatis and associates (9). Restriction enzymes, T4 DNA ligase, and the Klenow large fragment of DNA polymerase were purchased from Amersham International, Amersham, United Kingdom. Calf intestine alkaline phosphatase was obtained from Boehringer GmgH, Mannheim, Federal Republic of Germany.

cDNA copies representing BTV double-stranded RNA segments L3 and M7 were released from their pBR322 cloning vector by *Pst*I digestion. Homopolymeric tails introduced to aid the cloning procedure were removed by limited *Bal* 31 exonuclease digestion. Genes lacking the dC-dG tails but retaining the complete coding sequence were cloned into baculovirus dual-transfer vector pAcVC3 via the pUC4 vector. This intermediate plasmid allowed putative clones to be sequenced after exonuclease treatment and also *Bam*HI sites to be generated at the termini of the inserted gene by use of the symmetrical polylinker of the plasmid. Since the L3 gene contained internal *Bam*HI and *Bgl*II sites, the M7 gene was inserted into the pAcVC3 vector first.

Monolayers of *S. frugiperda* cells were cotranfected with this dual-transfer vector DNA (pAcVC3.BTV-10.7.BTV-17.3) and wild-type AcNPV DNA by the standard method of calcium chloride precipitation (15). Recombinant baculoviruses generated by a homologous recombinant were selected by virtue of their polyhedrin-negative phenotype and plaque purified (2).

SDS-PAGE analysis and purification of expressed particles. *S. frugiperda* cells were infected at a multiplicity of 10 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) (7) and stained with Coomassie brilliant blue.

The expressed corelike particles were purified by lysing the cells as described above and banding either on a discontinuous sucrose gradient (30% to 50% [wt/vol] in 0.2 M Tris hydrochloride [pH 8.0]) after centrifugation at 85,000 × *g* for 3 h or on self-forming CsCl (35%) density gradients after centrifugaton at 140,000 × *g* for 18 h. The particles were analyzed by SDS-PAGE (as described above) or examined by electron microscopy. BTV particles and cores were purified from monolayers of BHK-21 cells infected with BTV serotype 10 as previously described (10).

Western blot (immunoblot) analysis. Proteins were separated by SDS-PAGE and electroblotted onto Immobilon (Millipore Corp., Bedford, Mass.) with a Sartorius semidry 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 Chemical Co., St. Louis, Mo.) and gently agitated at room temperature for 90 min. The filter was then washed again and placed in substrate (disodium β-naphthyl phosphate, Fast Blue BB Salt, Mg SO_4) for 5 min before being rinsed in water and dried.

Electron microscopy. *S. frugiperda* cells infected with the dual-recombinant baculovirus were harvested at 32 h postinfection and fixed in 2% glutaraldehyde (in 0.1 M phosphate buffer [pH 7.2]). After being washed in phosphate buffer, cells were fixed in 1% osmium tetroxide and then washed again and dehydrated with alcohol. Cells were embedded in EMIX resin, and sections were stained with 2% uranyl acetate and lead acetate.

Purified BTV-10 cores and expressed corelike 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 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.

Stoichiometry of VP3 to VP7. Monolayers of *S. frugiperda* cells were infected with the dual-recombinant baculovirus at a multiplicity of 10 PFU per cell and incubated at 28°C for 24 h. Radiolabeling of the viral proteins was achieved by incubating the cells in methionine-free medium for 1 h to reduce intracellular pools of this precursor and then adding 15 μCi of [^{35}S]methionine (>800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) per 10^6 cells. After 90 min, normal tissue culture medium was added and the incubation was continued for a further 12 h. The cells were then harvested and washed in phosphate-buffered saline and the expressed particles were purified on discontinuous sucrose gradients as already described. The particles were pelleted and mixed with dissociation buffer, and samples of various amounts were subjected to SDS-PAGE. After electrophoresis, the gel was stained with cold 0.25 M KCl and the bands of VP3 and VP7 were excised. Their activities (counts per minute) were determined after incubation for 12 h at 37°C in a liquid scintillation cocktail containing a tissue solubilizer. The experiment was performed twice, and the results were pooled.

RESULTS

Construction of a dual-recombinant baculovirus. cDNA copies representing the complete coding sequences of the BTV L3 and M7 genes were manipulated for expression in the baculovirus system as described in Materials and Methods (Fig. 1). Although the L3 and M7 genes were isolated from different BTV serotypes (BTV-17 and BTV-10, respectively), the L3 gene is highly conserved between these serotypes with an amino acid homology of greater than 99% (4). Homopolymeric tails introduced to aid the cloning procedures were removed by limited *Bal* 31 exonuclease digestion before the genes were inserted into the pAcVC3 transfer vector. 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 virus titers were determined by using confluent monolayers of *S. frugiperda* cells, and putative recombinants were selected on the basis of their

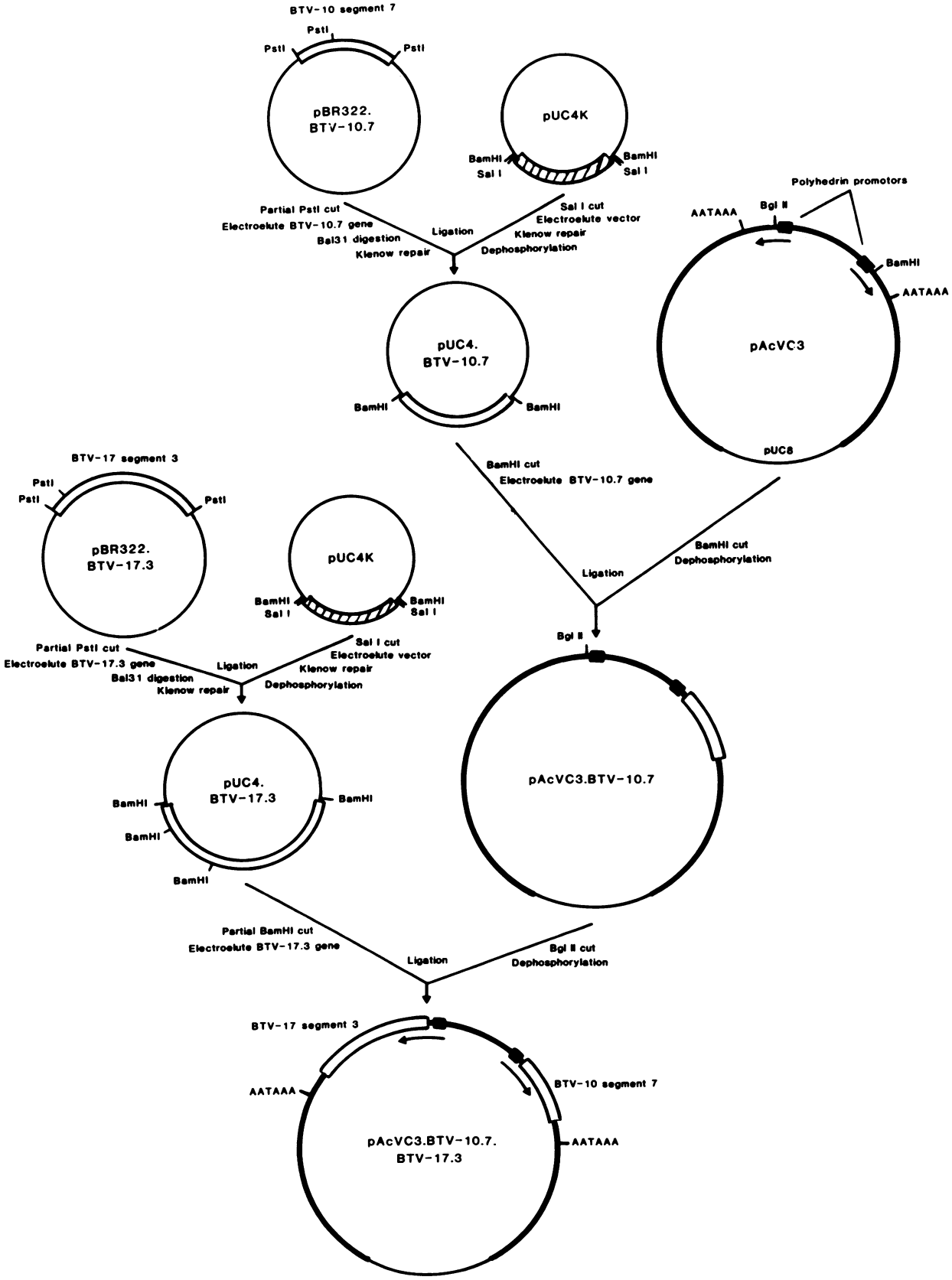

FIG. 1. Construction of the dual-expression transfer vector showing the appropriate manipulations for insertion of the BTV L3 and M7 genes.

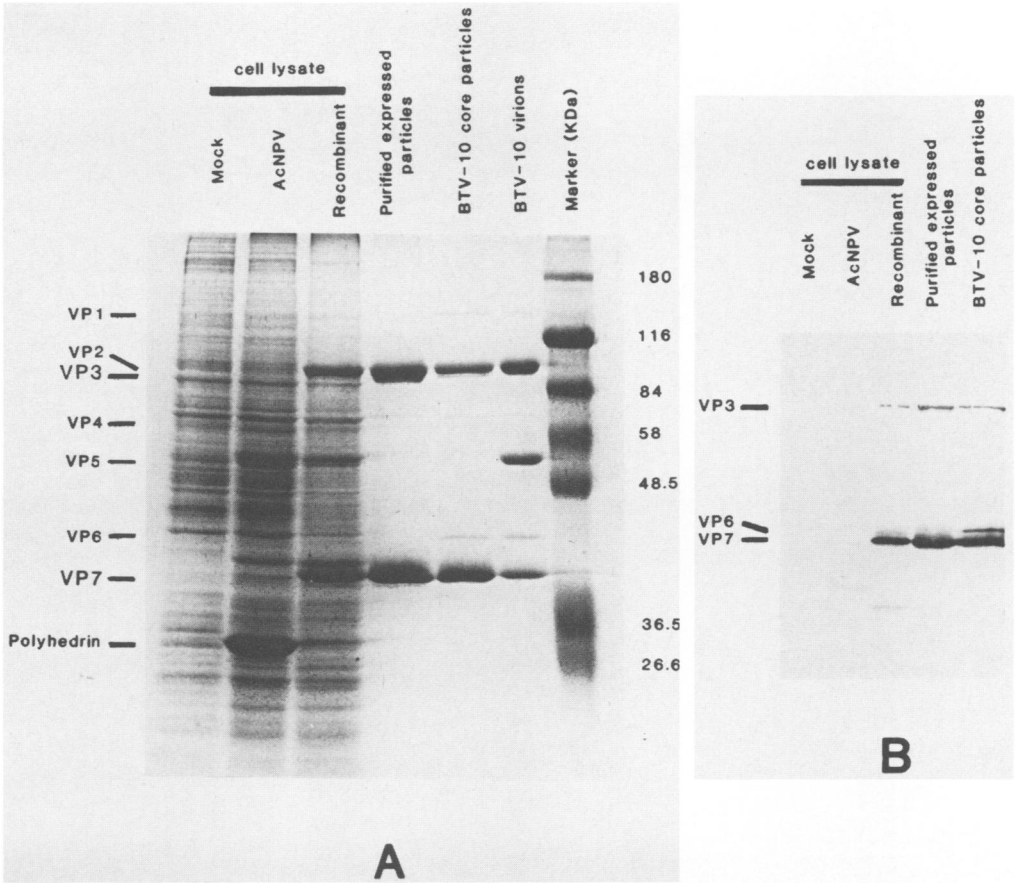

FIG. 2. Expression of BTV core proteins VP3 and VP7 in insect cells by the recombinant baculovirus and confirmation of their authenticity by Western immunoblot analysis. *S. frugiperda* cells were infected at a multiplicity of 10 PFU per cell with either the recombinant baculovirus or wild-type AcNPV or were mock infected. Cells were harvested at 48 h postinfection. The expressed particles were purified by banding on self-forming CsCl gradients. Authentic BTV virions and core particles prepared from BTV-infected BHK cells are included for comparison. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (A) or electroblotted onto Immobilon membrane and reacted with rabbit BTV-10 antiserum (B). Bound antibody was detected with an alkaline phosphatase conjugate by using standard methods. The serum contained antibodies to VP3, VP6, and VP7 but not antibodies to minor proteins VP1 and VP4. KDa, Kilodaltons.

polyhedrin-negative phenotype (ca. 0.1% frequency). After successive rounds of plaque purification, a high-titer viral stock was prepared.

Coexpression of VP3 and VP7 in *S. frugiperda* cells. In place of the 29-kilodalton polyhedrin protein seen in wild-type AcNPV-infected cells, *S. frugiperda* cells infected with the recombinant baculovirus synthesized two unique protein species which comigrated with VP3 and VP7 derived from purified BTV cores or virus particles (Fig. 2A). The sizes of the expressed proteins agreed with those expected for VP3 and VP7 on the basis of their amino acid compositions (i.e., 103,416 [13] and 38,548 [20] daltons, respectively).

Confirmation that the expressed proteins represented authentic BTV polypeptides was provided by Western immunoblot analyses with antiserum raised to BTV-10 virus particles (Fig. 2B). This antiserum reacted strongly with the VP3 and VP7 species in a cell lysate of *S. frugiperda* cells infected with the dual-recombinant baculovirus, while no reaction was detected with mock-infected or wild-type AcNPV-infected cells. Virion-derived BTV-10 core particles were included as a control, which showed that the BTV-10 antiserum also contained antibodies to minor core protein VP6 (but not to VP1 or VP4).

Electron micrographs of infected *S. frugiperda* cells and purified expressed particles. Electron micrographs of *S. frugiperda* cells infected with the recombinant baculovirus showed numerous aggregates of foreign material in both the cytoplasm and nuclei of the infected cells. Nonoccluded baculovirus particles were also visible within the nuclei (Fig. 3A). The aggregates consisted of spherical particles which, in many cells, were present as paracrystalline arrays (Fig. 3A, insert). The particles were isolated by lysing the infected cells with the nonionic detergent Nonidet P-40 and purification by centrifugation on discontinuous sucrose gradients or self-forming CsCl gradients. In CsCl, the particles banded at a density of 1.307 g/cm^3. When examined by electron microscope, the material was found to consist of corelike particles whose size and appearance were similar to those of authentic BTV core particles prepared from BTV-infected BHK cells (Fig. 3B and C). The expressed particles appeared darker, presumably because, being empty, they absorbed more stain. Phenol extraction of purified expressed particles and examination by optical density measurements or agarose gel electrophoresis failed to demonstrate the presence of nucleic acid.

The purified particles were analyzed by SDS-PAGE (Fig. 2A) and shown to contain both VP3 and VP7 in the same proportions as in BTV-10 core particles. As with the infect-

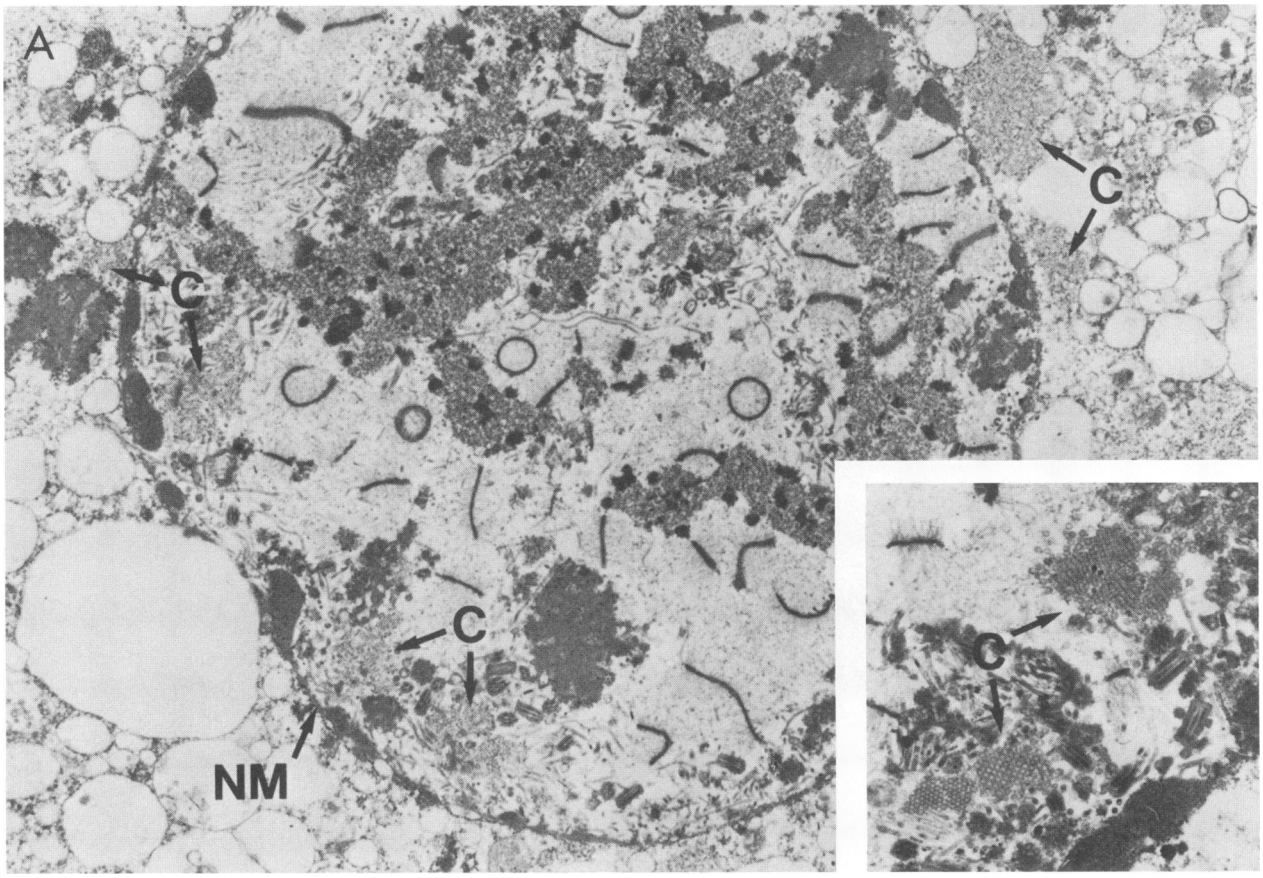

FIG. 3. Electron micrographs of empty BTV corelike particles synthesized in insect cells by a recombinant baculovirus expressing the two major BTV core proteins VP3 and VP7. (A) Section of an *S. frugiperda* cell infected with the recombinant virus (magnification, ×12,000). The insert shows the intranuclear paracrystalline arrays of expressed particles (magnification, ×20,000) present in many cells. Corelike particles (C) and the nuclear membrane (NM) are indicated by arrows. Expressed particles purified on self-forming CsCl gradients are shown in panel B, compared with authentic BTV core particles in panel C. Bar, 100 nm.

ed-cell lysates, BTV-10 antiserum reacted strongly with the VP3 and VP7 of purified particles in Western immunoblot analyses (Fig. 2B). Immunogold studies of the expressed particles produced the same results as with authentic viral cores (6), namely, that the particles could be heavily labeled with anti-VP7 serum but were not labeled with anti-VP3 serum (T. Collen, J. N. Borroughs, J. Anderson, R. Butcher, T. French, and P. P. C. Mertens, manuscript in preparation). The sedimentation coefficient of the expressed particles (225S) was approximately half that of BTV core particles derived from virus (470S) (5). This presumably reflects the absence of the double-stranded RNA genome of BTV. The yields of purified material were on the order of 30 mg/liter (1.5×10^9 cells).

Stoichiometry of VP3 to VP7 in expressed corelike particles. To appraise the relative proportion of VP3 to VP7 in the expressed particles more accurately, their stoichiometry was determined and compared with that of BTV-10 cores obtained from infectious BTV. *S. frugiperda* cells infected with the recombinant baculovirus were pulse-labeled at 24 h postinfection with [^{35}S]methionine, and after purification on discontinuous sucrose gradients, the labeled particles were dissociated and the protein components were separated by SDS-PAGE. The bands of VP7 and VP3 were located by autoradiography, excised, and counted in a liquid scintillation cocktail containing a tissue solubilizer. Since the number of methionine residues in each protein is known from sequence data, their stoichiometry was calculated. After the results from two different experiments were averaged, a value of 15 VP7 molecules to 2 VP3 molecules was obtained. This matches the stoichiometry of these proteins in BTV core particles as determined by labeling with ^{14}C-labeled amino acids (19).

DISCUSSION

Cryoelectron microscopy studies and immunogold analyses have indicated that the core of BTV consists of a nuclear-protein center surrounded by two distinct protein layers; VP7 forms the principal component of the outer layer, while VP3 is the major component of the inner layer. The location of the minor core proteins has not been mapped. In the present work, we sought to assemble single-shelled particles from VP3 and VP7 by constructing a dual-recombinant baculovirus that simultaneously expresses both proteins in *S. frugiperda* cells. Expressed VP3 and VP7 were found to interact spontaneously to form corelike particles of the same size, appearance, and stoichiometric arrangement of VP3 to VP7 as in authentic BTV cores. Further evidence that the expressed particles are accurately assembled was provided by immunogold analysis in which the particles were gold labeled in an identical manner to

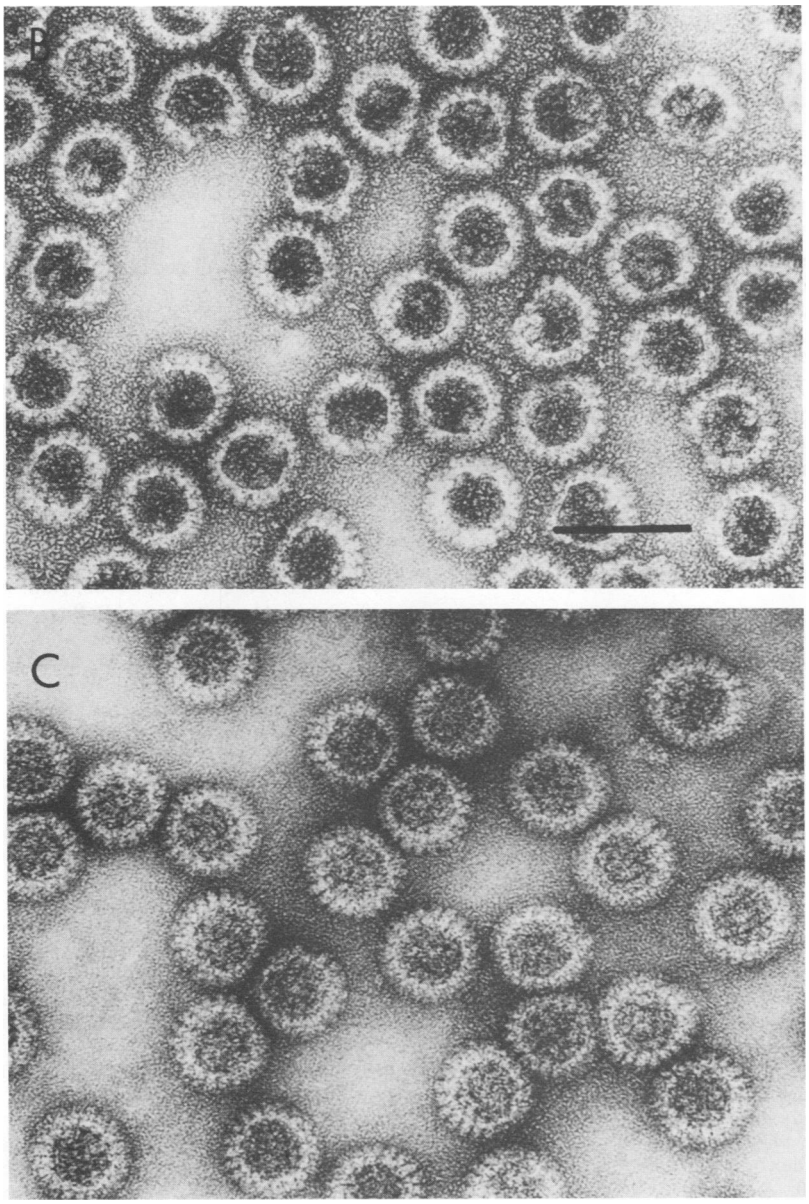

FIG. 3—*Continued.*

authentic cores. The corelike particles were present in both the cytoplasm and nuclei of infected insect cells. The reason for their intranuclear location is unknown, although since the electron micrographs were of cells harvested late in the infection cycle, degeneration of the nuclear membrane may have been a causative factor.

Assembly of these corelike particles from VP3 and VP7 demonstrated that the structural integrity of the core is provided by these protein species and that their formation in insect cells is not dependent on the presence of BTV double-stranded RNA or the minor core proteins. In addition, it can be concluded that the BTV nonstructural proteins are not required to either assist or direct the assembly of these empty particles. In view, however, of the apparent ease with which these corelike particles assemble it seems likely that the minor proteins and/or nonstructural proteins play a role in synchronizing the formation of complete core particles containing the full complement of proteins and 10 segments of double-stranded RNA. The formation of large amounts of empty corelike particles consisting only of VP3 and VP7 would obviously be unfavorable during BTV infection.

Recent evidence from our laboratory (Ventakaram Prasad et al., in preparation) indicates that the BTV core particle, like that of rotaviruses (18), has icosahedral symmetry with a triangulation number of 13. This symmetry indicates that 780 copies of VP7 are present per particle, which, based upon a 15:2 molecular arrangement, indicates that the core contains 104 copies of VP3. This 15:2 stoichiometric arrangement of VP7 to VP3 calculated for both BTV cores and expressed corelike particles makes it difficult to envisage their symmetry. If VP3 were attached at points of fivefold symmetry, as seems likely in physical terms, the ratio of VP7 to VP3 should be 13.2. Alternatively, if VP3 were bound at

points of five- and sixfold symmetry, the ratio would be approximately 12:2. The method used to calculate the stoichiometry of VP3 to VP7 in both authentic and expressed particles involved labeling the proteins with radioactive amino acids, dissociating the core, and measuring the activities of the component proteins. This technique obviously has limited accuracy. We are currently attempting to determine the molecular weight of the expressed particles and calculate from this the ratio of VP3 to VP7. This is possible because the molecular weights of VP3 and VP7 are known and the core contains 780 molecules of VP7, as determined by three-dimensional structural analyses of the BTV core by cryoelectron microscopic and image-processing techniques.

The assembly of BTV corelike particles following coexpression of VP3 and VP7 in insect cells provides an opportunity for more detailed research into BTV viral morphogenesis and the interactions of the component structural proteins. On a more general basis, the proven success of multiple-expression systems allows multimolecular structures containing nonequimolar ratios of proteins to be examined, as well as providing a means of testing antiviral reagents that may inhibit morphogenetic pathways.

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