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Identification of Bluetongue Virus VP6 Protein as a Nucleic Acid-Binding Protein and the Localization of VP6 in Virus-Infected Vertebrate Cells

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Recently the insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been effectively adapted as a highly efficient vector in insect cells for the expression of various genes. A cDNA sequence of RNA segment 9 of bluetongue virus serotype 10 (BTV-10, an orbivirus member of the *Reoviridae* family) encoding a minor core protein (VP6) has been inserted into the *Bam*HI site of the pAcYM1 transfer vector derived from AcNPV. *Spodoptera frugiperda* cells were cotransfected with the derived vector in the presence of authentic AcNPV DNA to produce recombinant viruses. These synthesized significant amounts of a protein (representing ca. 50% of the stained cellular protein) similar in size and antigenicity to the authentic BTV VP6. The expressed protein was identified as a nucleic acid-binding protein by using an RNA overlay-protein blot assay. A polyclonal anti-VP6 serum prepared by using the expressed VP6 protein has been used in an immunogold procedure to locate VP6 in BTV-infected mammalian cells. Gold was found to be associated with the matrix of virus inclusion bodies (VIB), with viruslike particles in the VIB, as well as with mature virion particles that were in close proximity to the VIB or were released from cells and adsorbed to cell surfaces. The recombinant virus antigen has also been used to identify antibodies to different BTV serotypes in infected sheep sera, indicating the potential of the expressed protein as a group-reactive antigen for the diagnosis of BTV infections.

Bluetongue virus (BTV) is an arthropod-borne virus that is an economically important pathogen of certain ruminants (e.g., sheep) in many parts of the world. The virus, which is spread by *Culicoides* vectors, induces a wide range of clinical and subclinical symptoms in sheep and cattle. The virus is a member of the *Orbivirus* genus of the *Reoviridae* family and is the best-studied virus at the genetic and molecular level among the 13 serogroups of orbiviruses that have been described.

The inner core of BTV is composed of five proteins, two major (VP3 and VP7) and three minor (VP1, VP4, and VP6) species, as well as the double-stranded RNA genome that consists of 10 discrete segments. This inner core is, in turn, surrounded by an outer capsid consisting of two proteins, VP2 and VP5 (23, 24). The inner core is 68 nm in diameter and exhibits icosahedral symmetry with a triangulation number of 13 (unpublished data). Preliminary examination of three-dimensional maps of the core particles indicates that there are three layers; the outermost 10-nm layer contains VP7, whereas VP3 is the major constituent of the 9-nm middle layer. In virus-infected cells parental BTV virions are converted to core particles, which are subsequently uncoated to subcore particles lacking only VP7 (10). These subcore particles are unstable and are composed of the viral genome encapsidated in a hexagonal protein shell, the majority of the protein mass consisting of VP3 with small quantities of the three minor proteins (VP1, VP4, and VP6). It is likely that the innermost part of the core consists only of the minor proteins and the viral RNA species. However, no data on the definite location or biophysical properties and functions of these minor proteins are available. To elucidate the structure-function relationships of the various BTV gene products, we have synthesized several of these core proteins by using baculovirus expression vectors (20). This paper describes the characterization of VP6 synthesized in *Spodoptera frugiperda* insect cells infected with a recombinant baculovirus and studies which identify BTV VP6 in BTV-infected mammalian cells.

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MATERIALS AND METHODS

Cells and virus. *S. frugiperda* cells were propagated either as suspension or as monolayer cultures in medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) at 28°C. A recombinant baculovirus containing the cDNA of the entire coding sequence of RNA segment 9 (S9) of BTV serotype 10 (BTV-10) driven by a polyhedrin promoter was produced by cotransfection of plasmid pAcYM1/BTV10-9 (Fig. 1) with wild-type AcNPV DNA in *S. frugiperda* cells (4, 15). A recombinant baculovirus (AcBTV10-9) was isolated, plaque purified, and propagated as previously described (6, 22).

DNA manipulation and recombinant plasmid construction. Standard DNA manipulation techniques were used to construct recombinant plasmids (14). Restriction enzymes, T4 DNA ligase, and the Klenow large fragment of DNA polymerase were purchased mainly from Amersham International, Amersham, United Kingdom. Calf intestine alkaline phosphatase was obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany.

A 1-kilobase DNA fragment containing the entire coding region of the BTV-10 S9 DNA was excised from a pBR322 plasmid (7) by *Nde*I and *Bam*HI digestion, repaired with the Klenow large fragment of DNA polymerase, and then cloned into the symmetrical polylinker of plasmid pUC4K (Fig. 1). The coding sequence of BTV-10 S9 DNA was then inserted

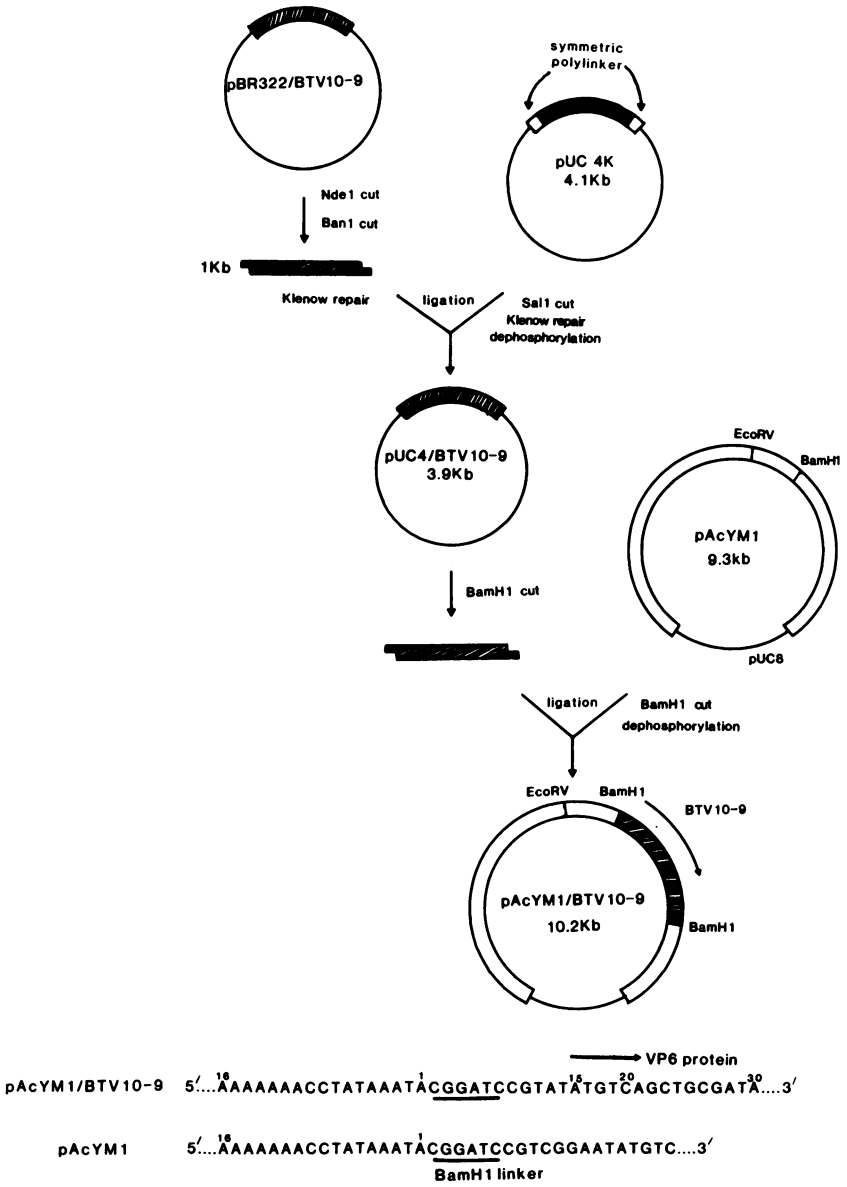

FIG. 1. Schematic diagram of the construction of the transfer vector. The full-length S9 of BTV-10 DNA was excised from a plasmid (pBR322/BTV10-9) by *Nde*I and *Bam*HI digestion and subcloned into pUC4K subcloning vectors. A candidate plasmid containing BTV S9 was then ligated to the *Bam*HI site of pAcYM1 vector as described in the text. Subsequently, a recombinant transfer vector (pAcYM1/BTV10-9) containing BTV-10 S9 DNA was selected. The sequence of the 5′ insertion site was determined by the method of Maxam and Gilbert (16).

into the *Bam*HI site of the transfer vector pAcYM1 as described previously (22).

A recombinant plasmid (10.2 kilobases) containing the insert in the correct transcriptional orientation was isolated and designated pAcYM1/BTV10-9. This transfer vector was used to generate recombinant baculoviruses through transfection and homologous recombination as described above.

Viral DNA purification and Southern analysis. *S. frugiperda* cells were infected with viruses at a multiplicity of 10 PFU per cell and incubated at 28°C for 3 days. The supernatant medium was harvested and centrifuged at 4,000 rpm for 10 min to remove cellular debris, and the virus was pelleted at 25,000 rpm for 1 h. The pellets were suspended in 1 ml of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 7.5]) and loaded onto the top of a sucrose gradient consisting of 5 ml of 50% (wt/vol) sucrose overlaid with 5 ml of 10% (wt/vol) sucrose in TE buffer. After centrifugation at 25,000 rpm for 1 h, the viral particles were recovered from the gradient interface and diluted with 4 volumes of TE buffer. After being repelleted at 25,000 rpm for 1 h, the virions were resuspended in TE buffer and disrupted by the addition of 0.2 volume of 10% sodium *N*-lauryl sarcosinate–10 mM EDTA (pH 7.5). The sample was then incubated at 60°C for 10 min and phenol extracted, and the nucleic acids were recovered by ethanol precipitation. For Southern analyses DNA samples were digested with *Bam*HI, and the products were resolved by electrophoresis in 1.0% agarose and then transferred by blotting to Hybond-N membrane (Amersham). After being dried and fixed with UV light, DNA was hybridized to a nick-translated BTV-10 S9 DNA

insert obtained from clone pAcYM1/BTV10-9, and the membrane was washed, dried, and autoradiographed (21).

BTV purification. Monolayers of BHK-21 cells were infected with U.S. BTV-10 at a multiplicity of 5 PFU per cell and incubated for 36 to 40 h at 37°C. Intact virus particles and cores were purified by using methods described by Mertens et al. (17).

Analysis of infected-cell polypeptides by SDS-PAGE. *S. frugiperda* cells in 35-mm tissue culture dishes were infected with viruses at a multiplicity of 10 PFU per cell, and the cells were incubated at 28°C for 4 days. At the end of the incubation period, the cells were rinsed three times with phosphate-buffered saline (PBS) and suspended in 100 μl of 10 mM Tris hydrochloride buffer (pH 7.4). A 50-μl volume of protein dissociation buffer (2.3% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris hydrochloride, 0.01% bromophenol blue [pH 6.8]) was added to each sample, and the mixture was heated at 100°C for 10 min. Partially purified VP6 was prepared from infected-cell extracts by using sucrose gradient (10 to 50% [wt/vol] in TE buffer) centrifugation as described previously (22). The majority of the expressed VP6 remained in the top of the gradient. Proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) in a 10 to 20% linear gradient polyacrylamide gel in the presence of SDS as described by Laemmli (12). After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue.

Immunoblot analyses. The procedure for immunoblotting the sample was similar to that described previously (22). In short, *S. frugiperda* cells infected with the recombinant baculovirus or wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV), or purified BTV-10 cores prepared from BHK-21 cells were separated by gradient PAGE (as described above) and blotted onto an Immobilon filter (Millipore Corp., Bedford, Mass.) by using a Sartorius "semi-dry" electroblotter. The filter was soaked overnight at 4°C in blocking buffer (5% skim milk plus 0.05% Tween-20 in PBS), hyperimmune rabbit anti-BTV-10 serum (1:1,000 dilution in blocking buffer) was added, and the filter was gently agitated for 90 min at room temperature. After multiple washing with 0.05% Tween 20 in PBS, the filter was returned to blocking buffer containing a 1:1,000 dilution of goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) and gently agitated at room temperature for 90 min. After further washing with 0.05% Tween 20 in PBS, bound antibodies were detected by incubation in a mixture of Fast BB salt and β-naphthyl phosphate (Sigma) as a substrate. To detect group-specific antigenicity, blots were prepared with a mixture of partially purified recombinant VP6 and purified NS1 protein (22) and incubated with anti-BTV-10 antisera or with anti-EHDV-1 or anti-EHDV-2 sheep sera. Each antiserum was diluted 1:20 to 1:500. Bound antibodies were detected as above.

Preparation of antibodies to the expressed VP6. *S. frugiperda* cells were infected with AcBTV10-9 and harvested at 3 or 4 days postinfection. Approximately 10 mg of expressed VP6 was resolved by SDS-PAGE, and the SDS-protein complexes were identified by precipitation with KCl. The band containing VP6 protein was excised and crushed in PBS, and the resulting slurry was injected intraperitoneally into a rabbit at days 0, 7, 14, and 21. Serum samples were removed at 2-day intervals from days 22 to 30. Each sample was tested for antigenicity, and the serum with high titer was cleaned by absorption with an acetone powder extract of *S. frugiperda* cells.

Immunogold labeling of VP6 antibody and electron micrographs of infected cells. BHK-21 cells were infected with BTV-10 at a multiplicity of 0.1 PFU/ml and incubated at 37°C for 3 days. The cells were washed with PBS and fixed with 2.5% glutaraldehyde. Samples were dehydrated with ethanol and embedded in L.R. White resin (London Resin Co.) as described by Newman et al. (18). Ultrathin sections were then prepared and mounted on uncoated nickel grids. Uninfected cells were similarly prepared for control experiments. For gold-labeling experiments, grids were preincubated in 0.1% bovine serum albumin in PBS for 10 min and then incubated for 2 to 3 h with rabbit VP6 serum diluted in 0.1% bovine serum albumin in PBS. The grids were then washed three times with PBS and then once with 0.1% bovine serum albumin in PBS. The sites of antibody binding were determined by incubating the grids with colloidal gold particles (diameter, 10 nm) conjugated to goat anti-rabbit immunoglobulin G (BioCell Research Laboratories). For controls, preimmune rabbit serum was used for both BTV-infected and uninfected cells. The bound gold particles were identified by using a JEOL electron microscope.

Preparation of single- and double-stranded nucleic acid probes. For the preparation of a single-stranded RNA probe, the DNA insert of a viral RNA segment (segment 10 of BTV-10) was recovered from the plasmid transfer vector by *Bam*HI digestion (6) and ligated to the dual promoter vector pST-18 plasmid (Pharmacia Inc., Piscataway, N.J.) containing the SP6 and T7 promoters. A clone containing the viral DNA insert in the correct orientation for the SP6 promoter was selected following restriction enzyme mapping. For in vitro transcription of the pSPT-18–BTV10-10 construct, plasmid DNA was purified by CsCl gradient centrifugation as described above and then linearized (downstream of the insert) by *Eco*RI digestion. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation. By using bacteriophage SP6 RNA polymerase (Pharmacia), [α-^{32}P]UTP (Amersham), and 1 μg of linear DNA templates, we generated ^{32}P-labeled RNA runoff transcripts (2×10^6 cpm/g) in vitro in a reaction mixture containing 400 μm of the other three nucleoside triphosphates, 1 mM dithiothreitol, 40 mM Tris hydrochloride (pH 7.9), 6 mM $MgCl_2$, and 2 mM spermidine. After 1 h at 37°C, 5 U of RNase-free DNase I was added, and the incubation was continued for a further 10 min. The single-stranded RNA transcripts were then recovered by phenol extraction, chloroform extraction, and ethanol precipitation. The single-stranded nonviral RNA probe was similarly prepared from pST-18.

BTV-10 double-stranded RNA species were purified from infected BHK-21 cells as described by Ritter and Roy (19), dephosphorylated with calf intestine alkaline phosphatase, and labeled with T4 polynucleotide kinase in the presence of 60 μCi of [γ-^{32}P]ATP as described by Maniatis et al. (14).

For the preparation of ^{32}P-labeled DNA probes, the double-stranded DNA was purified from pUC19 and nick translated in the presence of [α-^{32}P]dATP.

Nucleic acid-protein binding assay. Partially purified VP6 was prepared from infected cells through sucrose gradient centrifugation as described above. Proteins were then analyzed by SDS-PAGE and transferred to an Immobilon filter by using an electroblotter as described above. The filter was incubated in standard binding buffer (SBB; 50 mM NaCl, 1 mM EDTA, 10 mM Tris hydrochloride [pH 7.0], 0.02% Ficoll [Pharmacia], 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) at room temperature for at least 1 h before the probes were added. The individual filter was incubated with each of the probes at room temperature for 1

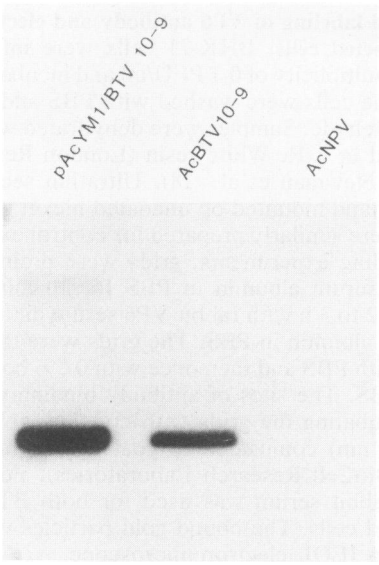

FIG. 2. Southern blot analyses of recombinant baculovirus nucleic acids. The *Bam*HI digests of DNA recovered from transfer vector pAcYM1/BTV10-9, wild-type AcNPV, and recombinant virus pAcBTV10-9 were resolved by agarose gel electrophoresis and probed with nick-translated BTV-10 S9 DNA.

h. The unbound probes were removed by washing the filters twice with SBS for 20 min at room temperature and then once at 37°C. Each filter was then dried and autoradiographed.

RESULTS

Construction of recombinant viruses. The complete coding region of a DNA copy of BTV-10 S9 was recovered from the cloning vector pBR322 and inserted into the transfer vector pAcYM1 (15) as described in Materials and Methods. To remove unnecessary sequences (such as homopolymeric tails), we used the restriction enzymes *Nde*I and *Bam*HI to isolate the coding region together with 5 base pairs upstream of the translation initiation ATG codon and 42 base pairs downstream of the TAG translation termination codon (7). The orientation and junction sequences of the transfer vector were confirmed by sequence analyses (16) (Fig. 1).

The BTV S9 gene was inserted into the AcNPV genome by cotransfecting *S. frugiperda* cells with pAcYM1/BTV10-9 DNA and infectious wild-type AcNPV DNA. Plaques exhibiting a polyhedrin-negative phenotype were selected, and viral DNA was prepared after further plaque purification. The presence of the S9 gene in the recombinant virus (designated AcBTV10-9) was demonstrated by Southern analyses following *Bam*HI digestion of viral DNA (Fig. 2). As expected, the nick-translated BTV10-9 probe did not hybridize to wild-type AcNPV DNA, but it did hybridize to AcBTV10-9 DNA and to the *Bam*HI-excised DNA obtained from the transfer vector pAcYM/BTV10-9.

Expression of BTV-10 VP6 in *S. frugiperda* cells. To demonstrate that BTV-10 VP6 was synthesized in the recombinant baculovirus-infected cells, we prepared protein extracts from *S. frugiperda* cells infected with the recombinant AcBTV10-9 as described in Materials and Methods. Samples were also prepared from wild-type AcNPV- and mock-infected cells. Each sample was then resolved by SDS-PAGE, and protein bands were identified by staining with Coomassie brilliant blue (Fig. 3A).

In comparison with the 33-kilodalton polyhedrin protein induced by AcNPV (8) (Fig. 3A, lane 2), the recombinant virus synthesized a unique protein with an approximate size of 40 kilodaltons (Fig. 3A, lane 3), similar in size to that predicted from the amino acid sequence of the S9 gene

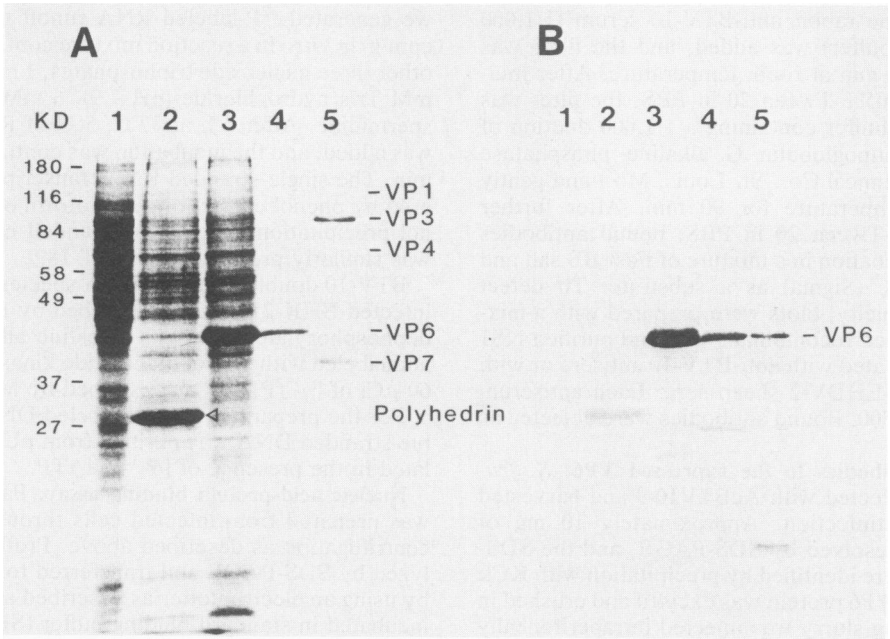

FIG. 3. Expression of VP6 in *S. frugiperda* cells by a recombinant baculovirus AcBTV10-9. Protein samples were resolved by 10 to 20% linear gradient SDS-PAGE and stained with Coomassie brilliant blue (A). Blotted Immobilon transfer membrane was reacted with anti-BTV-10 rabbit serum by using an alkaline phosphatase conjugate detection procedures (Western blot) (B). Lanes: 1, mock-infected *S. frugiperda* cell lysate; 2, wild-type AcNPV-infected *S. frugiperda* cell lysate; 3, AcBTV10-9-infected *S. frugiperda* cell lysate; 4, sucrose gradient-purified VP6; 5, purified BTV-10 core particles. Molecular masses in kilodaltons (kD) are shown on the left of panel A.

product (7). VP6 appears to be the major protein synthesized by the recombinant virus. In contrast, purified BTV-10 core particles contain only minute amounts of VP6 (Fig. 3A, lane 5). To estimate the approximate amounts of the protein synthesized by the recombinant virus, different concentrations (0.5 to 5 μg) of bovine serum albumin were electrophoresed in parallel with the extracts of cells infected with the recombinant virus (data not shown) and the protein bands were scanned in a densitometer. By this means, the putative VP6 was estimated to be expressed at approximately 100 mg/liter of infected cells (2×10^9 cells). The partially purified VP6 obtained by sucrose gradient centrifugation (see Materials and Methods) (Fig. 3A, lane 4) showed that most of the cellular proteins could be easily eliminated from the preparation.

To confirm that the 40-kilodalton protein was indeed BTV-10 VP6, a sample of each preparation was electrophoresed, transferred to an Immobilon filter, and subjected to Western immunoblot analyses with anti-BTV-10 serum as described in Materials and Methods. The strong positive signals (Fig. 3B, lanes 3 and 4) confirmed that the expressed 40-kilodalton band was indeed a BTV gene product.

Electron micrographs and localization of VP6 in BTV-infected cells. To confirm the presence of VP6 in BTV-infected cells and to delineate the nature of VP6 association with the virion particle, BTV-10-infected cells (at a multiplicity of 0.1) were prepared and incubated sequentially with anti-VP6 (1:100) monospecific rabbit serum (prepared by using expressed VP6 as the immunogen) and then with goat anti-rabbit immunoglobulin G conjugated to colloidal gold particles (diameter, 10 nm) as described in Materials and Methods. VP6 was identified in the infected-cell sections by the gold label (Fig. 4). The immunogold is able to bind only to the antigens that are exposed at the surface of the section. Since the tissues were embedded in plastic resin, not all visible structures were labeled uniformly. Nevertheless, the gold appeared to be localized not only in the virus particles identified in the cytoplasm, but also in the matrix of the virus inclusion bodies (VIB) as well as in the viruslike particles present within the VIB (Fig. 4A and C). Many virus particles were observed at the edge of the VIB (Fig. 4B) and in the cells (Fig. 4C). In contrast, the viral tubular structures were not associated with the gold particles, indicating that the tubular structures lack VP6 protein. No gold particles were observed when VP6 antiserum was substituted by preimmune serum; nor were they observed in mock-infected BHK-21 cells (data not shown).

Demonstration of the nucleic acid-binding capabilities of VP6. Sequence analyses have indicated that BTV-10 VP6 is rich in lysine and arginine residues. Since VP6 is a component of the viral nucleocapsid structure, it may be an RNA-binding protein (7). To address this question, we used a nucleic acid overlay–protein-blotting method (1, 3) as described in Materials and Methods. For the preparation of a ^{32}P-labeled single-stranded RNA probe, the cDNA copy of RNA segment 10 of BTV-10 was recloned into the dual-promoter vector and RNA was synthesized by using SP6 RNA polymerase as described previously (6). The partially purified VP6 was demonstrated to be a nucleic acid-binding protein as speculated. Both purified BTV proteins (Fig. 5, lane 1) and partially purified expressed VP6 samples were analyzed (Fig. 5, lanes 3 to 6) by this procedure. VP6 is capable of binding not only the double-stranded BTV RNA species (Fig. 5, lanes 1 and 3) and single-stranded BTV RNA transcripts (lane 4), but also a nonviral double-stranded DNA probe (lane 5) and a nonviral single-stranded RNA probe (lane 6). The weak positive signals with VP6 derived from BTV-10 (lane 1) reflect the amount of VP6 present in virions, confirming that it is indeed a minor component of the particle.

Demonstration of the group-specific antigenicity of VP6. Since Northern (RNA) blot hybridization data (19) have indicated that BTV S9 is conserved among most BTV serotypes, it was of interest to determine whether VP6 represented a group-specific antigen. To compare the specificity to another BTV antigen, a second BTV gene product, the NS1 protein, was prepared (22) and mixed with VP6, and the mixture of the two proteins was resolved by gel electrophoresis. Blots of the proteins were prepared and incubated with different BTV antisera (i.e., anti-BTV-10, or anti-BTV-11, anti-BTV-13, or anti-BTV-17) as well as with antisera obtained from sheep immunized with epizootic hemorrhagic disease virus (EHDV-1 and EHDV-2), a related orbivirus. Strong positive reaction signals were detected with all the BTV antisera tested (Fig. 6). The weak reactions detected with EHDV-1 (Fig. 6, lane 10) and EHDV-2 (lane 11) antisera were similar to those obtained with normal sheep sera (lanes 4 and 5), indicating nonspecific reactions. The results of Western blot analyses for the NS1 proteins of BTV-10 were quite different. The BTV-10 NS1 protein reacted with both BTV-10 and BTV-11 antisera when the antisera were prepared by using partially purified or nonpurified virus (Fig. 6, lanes 1, 6, and 7). However, unlike VP6, no reaction was observed with BTV antisera that had been prepared by using purified virions (lane 2), indicating that the NS1 protein is not associated with purified virus particles. Neither anti-BTV-13, anti-BTV-17, anti-EHDV-1, nor anti-EHDV-2 sera reacted with NS1 protein in the assay. In all the blots, a number of nonspecific smaller bands were also observed. These were probably degradation products of VP6. Such smaller polypeptide bands were also evident in the Coomassie blue-stained strip (Fig. 6, lane C).

DISCUSSION

Data are presented on the expression of BTV VP6 by a recombinant baculovirus containing a 1,031-base-pair insert of BTV-10 S9. It was estimated that the level of expression of VP6 was of the order of 100 mg/2×10^9 infected cells. This level of synthesis is considerably higher than that obtained in mammalian cells infected with BTV. The expressed protein in insect cells is similar to BTV VP6 in both size and antigenicity, as shown by Western blot analyses.

Until now, only NS2, one of the BTV nonstructural proteins, has been shown to be a nucleic acid-binding protein. However, from the sequence data of S9, VP6 was predicted to be a hydrophilic protein and the richest of all the BTV gene products in its content of basic and charged amino acids per unit length of 1,000 amino acids (7). We postulated that the VP6 molecules may be associated with BTV genomic RNA. We have demonstrated here that VP6 is indeed a nucleic acid-binding protein and that it is capable of binding virion double-stranded RNA, single-stranded RNA transcripts, and other nucleic acid species. It may be speculated that during viral morphogenesis, the de novo plus-strand RNA species binds with VP6 and, subsequently, full-length complementary minus strands are transcribed, rendering them double-stranded. It will be interesting to determine the precise binding site(s) of the protein for each RNA species.

Another feature of VP6 was revealed during this study. The protein was recognized by all the BTV antisera tested by

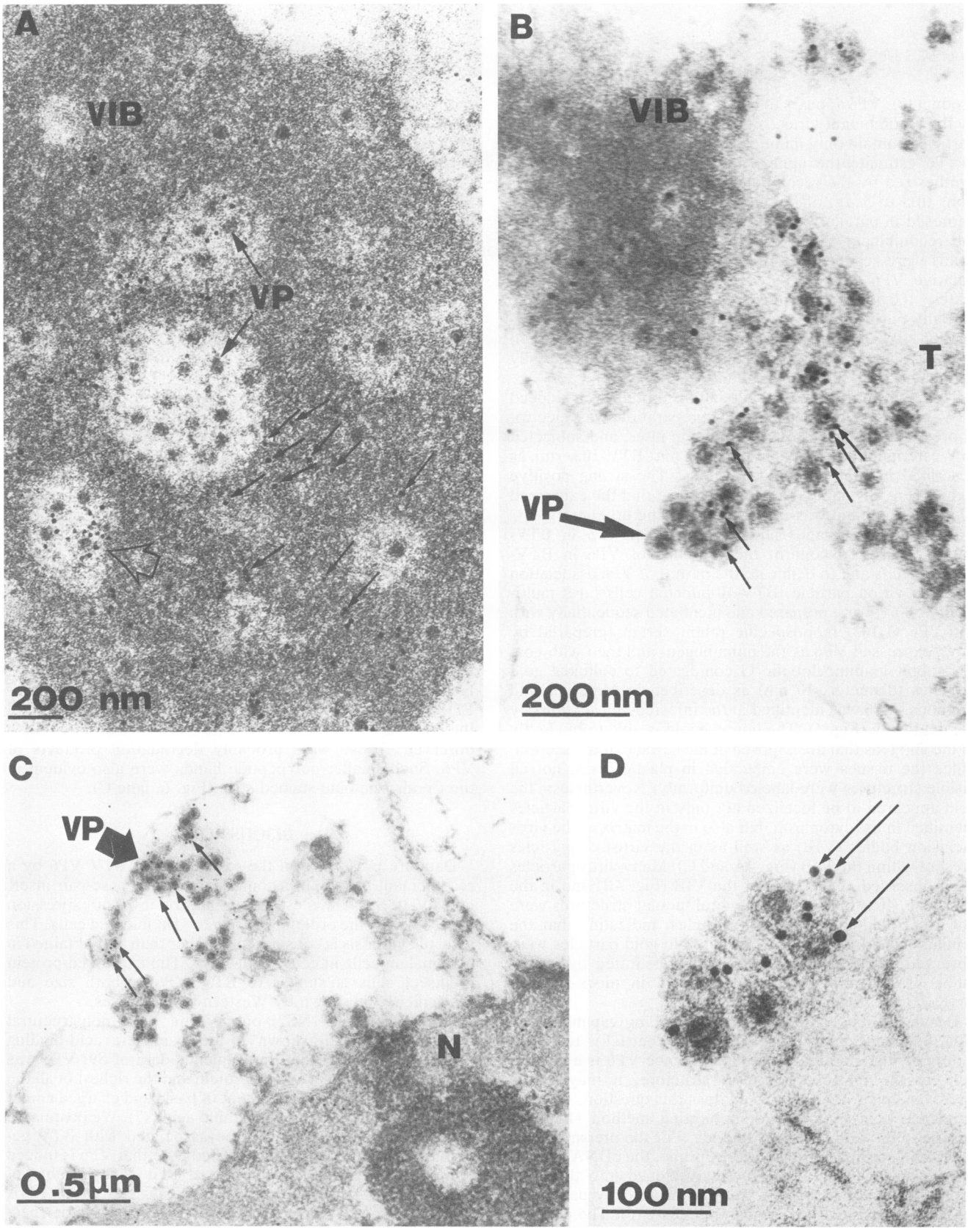

FIG. 4. Electron micrographs of thin sections of BTV-10-infected BHK-21 cells probed with anti-VP6 antibody labeled with gold particles. BTV-infected cells were fixed following infection and probed with monospecific VP6 antibodies as described in Materials and Methods. The antibodies were located by binding with the gold particles conjugated to goat anti-rabbit immunoglobulin G. (A) Gold labeling on VIB and in virus particles (VP) is indicated by small and large arrows, respectively. (B) Virus particles (VP) in the vicinity of the VIB. Gold particles are mostly on the virus particles (small arrows), but some are on the VIB. No particles were identified on the virus-induced tubular structures (T). (C) Virus particles (VP) outside the inclusion body in the process of release from a cell. N, Nucleus of the cell. Gold labeling of virus particles with VP6 antibody is indicated by small arrows. (D) Virus particles, labeled with gold particles, released from a cell and adsorbed to the cell surface.

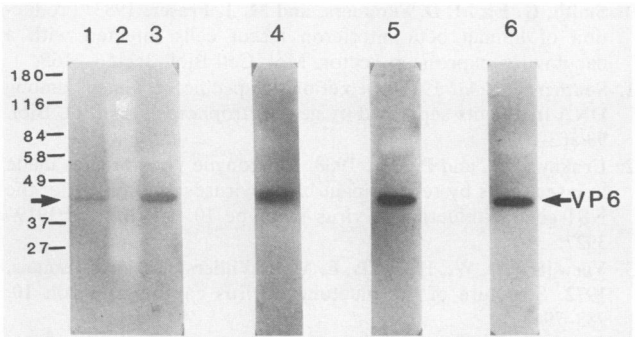

FIG. 5. Nucleic acids bind VP6 protein by the RNA overlay-protein blot assay. Proteins were analyzed by SDS-PAGE and transferred to an Immobilon filter. The protein blot was cut into strips, and individual strips were reacted with the double-stranded RNA probe (see Materials and Methods) (lane 3), BTV single-stranded RNA probe obtained by SP6 transcription of the BTV-10 segment 10 cDNA clone (lane 4), double-stranded DNA probe derived from pUC19 (lane 5), and single-stranded RNA probe derived by SP6 transcription of the pST-18 plasmid (lane 6). Lane 1 contains purified BTV-10 virion proteins, and lane 2 represents the marker protein mixture.

Western blot analysis. These results indicated that the minor core protein VP6 is conserved for all BTV serotypes, as expected. The antigen, therefore, has potential value for development as group-specific diagnostic reagents for screening of BTV antibody from BTV-infected blood samples. The expressed protein raised high-titer antisera (data not shown), indicating that it is very immunogenic.

In BTV-infected cells, virus morphogenesis is believed to occur within dense fibrillar cytoskeleton-associated structures called virus inclusion bodies (5, 13). Viral RNA has been detected by acriorange staining (2) within the inclusion bodies. These structures are assumed to be the sites for virus production and have recently been shown to contain the major core protein, VP7 (11). The data presented here demonstrate that the minor protein VP6 is also present within the VIB matrix. Anti-VP6 polyclonal antibodies labeled with colloidal gold particles reacted not only with the viruslike structure in the VIB of the thin section of BTV-infected cells but also with the matrix of VIB. As expected, significant numbers of gold particles were also present in association with mature virion particles. However, no gold particles were detected in the tubular structures characteristic of BTV-infected cells, structures consisting of the BTV protein NS1 (10, 22). The presence of VP6 in the VIB is consistent with a role for this protein in binding to single-stranded and double-stranded BTV RNA species during virus morphogenesis.

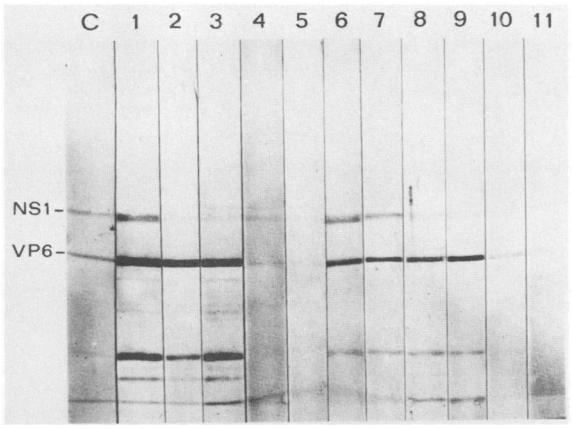

FIG. 6. Detection of group specificity of VP6 by using Western analyses. Semipurified VP6 and purified NS1 were resolved by gel electrophoresis and detected by Western analyses with different antisera as described in Materials and Methods. Each lane represents a separate Western analysis. Lanes: 1, anti-BTV-10 rabbit (immunized with crude virus) antiserum; 2, anti-BTV-10 rabbit (immunized with purified virus) antiserum; 3, anti-BTV-17 rabbit (immunized with purified virus) antiserum; 4 and 5, normal sheep serum; 6, anti-BTV-10 sheep serum; 7, anti-BTV-11 sheep serum; 8, anti-BTV-13 sheep serum; 9, anti-BTV-17 sheep serum; 10, anti-EHDV-1 sheep serum; 11, anti-EHDV-2 sheep serum; C, Coomassie blue-stained strip.

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