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Expression of Two Related Nonstructural Proteins of Bluetongue Virus (BTV) Type 10 in Insect Cells by a Recombinant Baculovirus: Production of Polyclonal Ascitic Fluid and Characterization of the Gene Product in BTV-Infected BHK Cells

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In vitro translation of bluetongue virus (BTV) double-stranded RNA in the rabbit reticulocyte lysate system has shown segment 10 (S10) to code for two related proteins, NS3 and NS3A. The presence of both products in vivo, however, has remained unconfirmed owing to the very low level of synthesis of the S10 gene product(s) in BTV-infected BHK cells. In the present work, a cDNA copy of BTV type 10 (BTV-10) S10 RNA was inserted into *Autographa californica* nuclear polyhedrosis baculovirus (AcNPV) in lieu of the 5′ coding region of the AcNPV polyhedrin gene. *Spodoptera frugiperda* cells infected with the recombinant baculovirus synthesized two polypeptides, which were shown to represent NS3 and NS3A by Western blot (immunoblot) and peptide map analysis. Antibodies raised to the expressed NS3 by immunization of mice detected both NS3 and NS3A in BTV-10-infected BHK cells but not in purified BTV-10 virus particles. In contrast to in vitro translation of BTV S10 RNA in which NS3 and NS3A are synthesized in equimolar amounts, NS3 was the principle product both in the baculovirus expression system and in vivo in BTV-infected cells. The results indicate the caution which should be exercised when using the rabbit reticulocyte lysate system to predict the pattern of protein synthesis from a gene with alternative start codons. The expressed NS3 and NS3A proteins reacted strongly with sera from sheep infected with homologous and heterologous BTV serotypes, suggesting that the S10 gene products are highly conserved group-specific antigens.

Bluetongue virus (BTV) is the prototype virus of the *Orbivirus* genus in the *Reoviridae* family. The 10-segment double-stranded RNA (dsRNA) genome of the virus is located in a core particle consisting of two major proteins, VP3 and VP7, and three minor proteins, VP1, VP4, and VP6. An outer capsid composed of two major proteins, VP2 and VP5, surrounds the core (25). Protein VP7 is the major group-specific antigen of the virus (5, 7, 8), while VP2 is the main determinant of serotype specificity and is the antigen recognized by neutralizing antibodies (8). Analysis of the coding assignments for the BTV genome has revealed that RNA segments L1, L2, L3, M4, M5, S7, and S9 code for the proteins VP1, -2, -3, -4, -5, -7, and -6, respectively. In addition to these structural proteins, BTV also codes for a variety of nonstructural proteins whose functions are less well characterized. To date, five such proteins have been identified and designated NS1, NS2, NS3, NS3A, and NSX. In vitro translation studies have shown that these proteins are coded by BTV segments M6 (NS1), S8 (NS2), S9 (NSX), and S10 (NS3, NS3A) (24). Proteins NS3 and NS3A have previously been called P8 and P8A, respectively (17).

The sizes of the NS3 and NS3A proteins and the mechanism by which they are derived from the same genomic segment have not been firmly established. The S10 gene products are present in very low levels in BTV-infected cells; thus, attempts to resolve such questions have utilized in vitro translation of purified BTV dsRNA in the rabbit reticulocyte lysate system. Using this approach, Mertens et al. (17) estimated that the BTV type 1 (BTV-1) NS3 proteins had sizes of between 15 and 20 kilodaltons (kDa), while more recently Gould (4) has reported them to be approximately 25 and 24 kDa. Van Dijk and Huismans (24), however, have estimated NS3 and NS3A from BTV-10 to be 28 and 25 kDa, respectively. Excision of the NS3 and NS3A bands from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequent peptide mapping (1) has revealed marked similarities between the peptide maps of the two NS3 proteins, suggesting that they are related, possibly translated from the same open reading frame or derived from a common translation product, with NS3A representing a truncated form of NS3 (17, 24). The nucleotide sequence of BTV-10 S10 RNA contains two possible initiation sites in the same open reading frame (13). The first AUG codon does not conform to the Kozak consensus sequence (10, 11) owing to the presence of a pyrimidine at position +4. It has been suggested (4) that the low-level synthesis of NS3 in vivo may be due to this factor. The second AUG codon, however, does conform to the consensus sequence, and it has further been suggested that inefficient recognition of the first AUG codon by scanning ribosomes results in their migration to the second codon and thus synthesis of NS3A. If translation was initiated from both sites, the estimated sizes of the polypeptides would be 25,572 and 24,020 Da, respectively.

The function(s) of the NS3 proteins is unknown, although the presence of a low-molecular-weight polypeptide seen during BTV purification led Mertens and associates (17) to suggest that they form a structural component in the outer capsid of the virions. The low concentration of the NS3 polypeptides in vivo has so far restricted any structural or functional analysis; indeed, the synthesis of these products in BTV-infected BHK cells is such that they cannot easily be

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distinguished even after radiolabeling. The presence of both proteins in vivo has, to date, only been ascertained by the comigration of appropriate protein species in infected cell lysates with the in vitro-translated products of S10 dsRNA. Insufficient NS3 and NS3A in vivo has prevented direct comparison of these proteins with their in vitro-translated counterparts. In the present work, we sought to resolve this problem by synthesizing the BTV S10 gene products in an expression system. The *Autographa californica* nuclear polyhedrosis baculovirus (AcNPV) vector was chosen in view of the reported high levels of foreign gene expression (15, 23). This report describes the synthesis and characterization of the BTV S10 gene products and confirms that NS3 and NS3A are related to each other and that both are present in BTV-infected cells, although in disproportionate amounts.

MATERIALS AND METHODS

DNA manipulation and construction of DNA clones. Plasmid DNA was manipulated by following the procedures described by Maniatis and associates (14). Restriction enzymes, T4 DNA ligase, and the Klenow large fragment of DNA polymerase were purchased from Amersham International plc (Amersham, Bucks, United Kingdom). Calf intestinal alkaline phosphatase was obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany). Denatured BTV S10 RNA species were polyadenylated at their 3′ ends, and cDNA copies were synthesized with reverse transcriptase in the presence of deoxyribonucleoside triphosphates and an oligo$(dT)_{12-18}$ primer as previously described (19). The RNA template strands were removed by RNase H treatment (14, 18), the cDNA products were self-annealed, and after tailing with dC, the duplexes were cloned into the *Pst*I site of pBR322. The complete nucleotide sequence of this clone has been previously reported by Lee and Roy (13).

Insertion of BTV-10 S10 DNA into the baculovirus transfer vector. The viral S10 cDNA insert was excised from pBR322 by *Pst*I digestion, and the terminal dC-dG sequences were removed by limited exonuclease digestion with *Bal* 31. Following repair with Klenow enzyme, the DNA was ligated into the dephosphorylated *Sma*I site of the transfer vector pAc373.S. Recombinant transfer vectors were isolated by restriction enzyme mapping, and after sequence analysis, a clone was chosen which lacked the dC-dG tails but retained the ATG translation initiation site. While these experiments were in progress, the high-level expression vector pAcYM1 was developed (15); therefore, the S10 cDNA was removed from the pAc373.S vector by *Bam*HI digestion and ligated into pAcYM1. The derived recombinants were isolated by colony hybridization, and a clone (pAcYM1.BTV-10.10) containing the viral DNA insert in the correct orientation was selected by restriction enzyme mapping and sequencing across the *Bam*HI insertion site (16) (Fig. 1).

Cotransfection of *Spodoptera frugiperda* cells and selection of recombinant viruses. AcNPV genomic DNA was purified by the method of Smith and Summers (22) and used to cotransfect *S. frugiperda* cells with the transfer vector pAcYM1.BTV-10.10 plasmid DNA by a modification of the procedures described by Smith et al. (23). A 1-μg sample of viral DNA was mixed with 25 to 100 μg of plasmid DNA and adjusted to 950 μl with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (20 mM HEPES buffer [pH 7.5], 1 mM disodium hydrogen P_i, 5 mM potassium chloride, 140 mM sodium chloride, 10 mM glucose). The DNA was precipitated with 50 μl of 2.5 M calcium chloride, and the resultant suspension was used to inoculate monolayers of 1.5×10^6 *S. frugiperda* cells in 35-mm tissue culture dishes. The DNA was allowed to adsorb to the cells for 1 h at room temperature, and then the supernatant fluid was discarded and replaced with 1.5 ml of medium containing 10% fetal bovine serum. After 4 days of incubation at 28°C, the supernatant fluid was harvested and titers of progeny viruses were determined with confluent monolayers of *S. frugiperda* cells. Plaques containing recombinant viruses were identified by their absence of occlusion bodies (polyhedra) when examined by light microscopy. Viruses from such plaques were replaqued twice more and used to prepare a high-titer polyhedrin-negative virus stock (10^8 PFU/ml).

Viral DNA purification and Southern analysis. *S. frugiperda* cells were infected with virus at a multiplicity of 10 PFU per cell and incubated at 28°C for 72 h. The supernatant medium was harvested and centrifuged at 5,000 × *g* for 10 min to remove cellular debris, and the virus was pelleted at 75,000 × *g* 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 (both prepared in TE buffer). After centrifugation at 100,000 × *g* for 1 h, the viral particles were recovered from the gradient interface and diluted with 4 volumes of TE buffer. After repelleting at 75,000 × *g* 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). After incubation at 60°C for 10 min, the sample was phenol extracted and the nucleic acids were recovered by ethanol precipitation. This procedure yielded DNA suitable for Southern analysis; however, when DNA was required for transfections, it was purified on a CsCl gradient at 100,000 × *g* for 18 h (22). 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 drying, DNA was hybridized to nick-translated BTV-10 S10 DNA obtained from clone pAcYM1.BTV-10.10, and the membrane was washed, dried, and autoradiographed (21).

RNA preparation and Northern (RNA) blot analysis of infected cell extracts. *S. frugiperda* cells were infected at a multiplicity of 10 PFU per cell and incubated at 28°C for 24 h. The cells were recovered by centrifugation and rinsed in phosphate-buffered saline (PBS). RNA was extracted by the guanidinium-hot phenol method (3), and polyadenylated RNA species were isolated with oligo(dT)-cellulose (14). RNA preparations were resolved by electrophoresis in 1% agarose containing 10 mM methylmercuric hydroxide, transferred to Hybond-N membrane, and hybridized to ^{32}P-labeled, nick-translated DNA probes as described by Denhardt (2). After hybridization, the membranes were washed and autoradiographed.

Viral protein and Western immunoblot analysis. Monolayers of *S. frugiperda* cells in 35-mm dishes were infected with wild-type AcNPV or recombinant viruses at a multiplicity of 10 PFU per cell and incubated at 28°C for 24 or 48 h. Radiolabeling of the viral proteins was achieved by initially incubating the cells in methionine-free medium for 1 h to reduce intracellular pools of this precursor and then adding 25 μCi of [^{35}S]methionine (>800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) per dish in methionine-free medium. The cells were incubated at 28°C for 2 h, harvested, rinsed three times with cold PBS, and lysed in 100

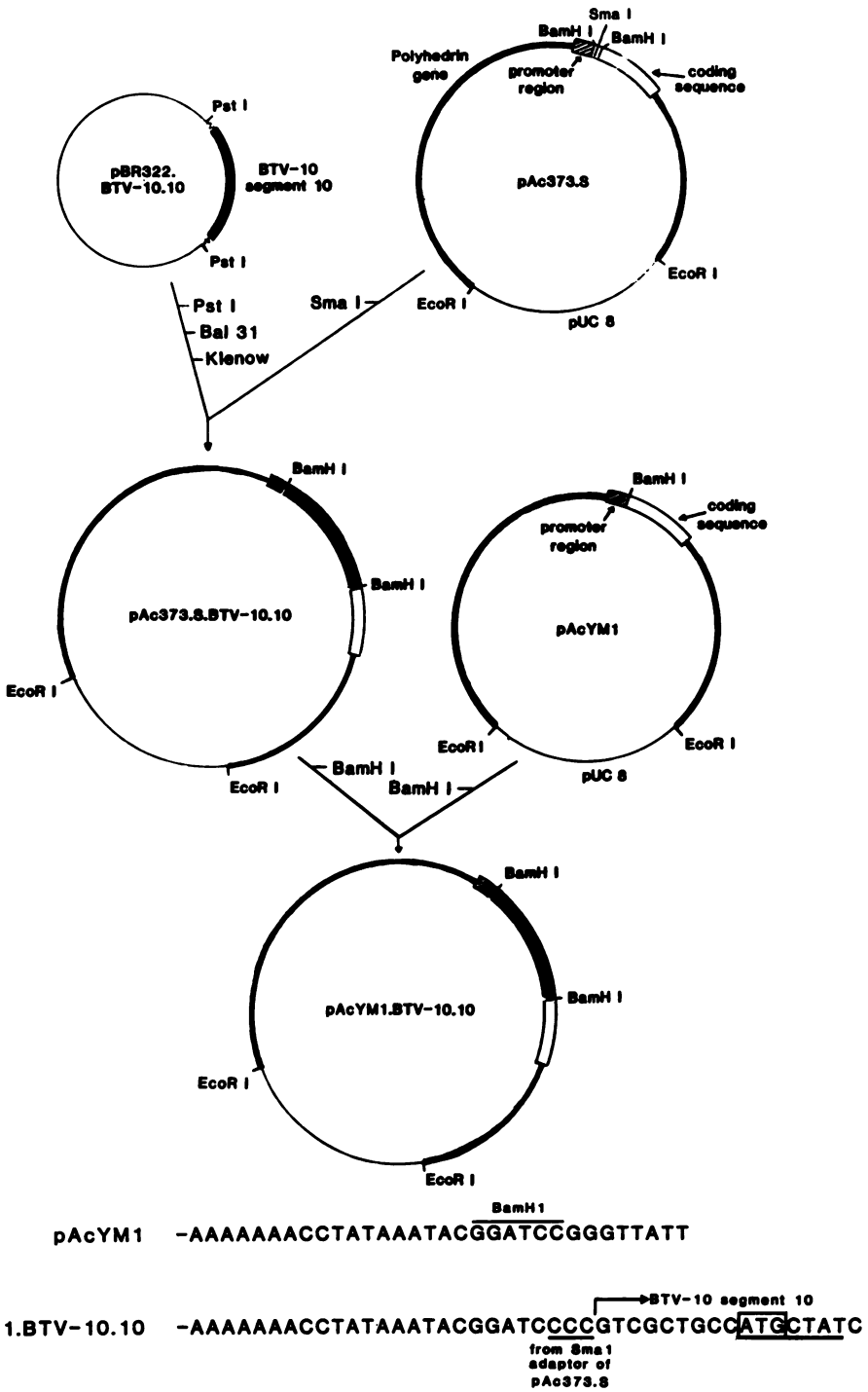

FIG. 1. Construction of the pAcYM1.BTV-10.10 recombinant baculovirus transfer vector. For details see Materials and Methods. The sequence at the 5′ insertion site of the pAcYM1.BTV-10.10 recombinant transfer vector is shown compared with that of the pAcYM1 transfer vector.

μl of RIPA buffer (1% Triton X-100; 1% sodium deoxycholate, 0.15 M NaCl, 0.01 M Tris hydrochloride, 0.01 M EDTA, 0.1% SDS, pH 8.0). BTV-10 polypeptides were obtained by infecting monolayers of BHK-21 cells at a multiplicity of 5 PFU per cell and incubating the cells at 35°C for 24 h. Proteins were labeled with [^{35}S]methionine by the procedure described above. Aliquots (20 μl) of the cell lysates were mixed with 20 μl of dissociation buffer (10% β-mercaptoethanol, 10% SDS, 25% glycerol, 10 mM Tris hydrochloride [pH 6.9], 0.02% bromophenol blue), heated to 100°C for 10 min, and electrophoresed in 8 to 20% gradient polyacrylamide slab gels prepared as described by Laemmli (12). After electrophoresis, the gels were stained with Coomassie brilliant blue R in 10% (vol/vol) aqueous acetic

acid containing 45% methanol. Radioactive gels were impregnated with Amplify (Amersham), dried, and exposed to X-ray film at −70°C.

For Western blot analysis, ^{35}S-labeled proteins from *S. frugiperda* cells infected with recombinant baculovirus or wild-type AcNPV or from BHK cells infected with BTV-10 were separated by gradient PAGE (as described above) and blotted onto a Durapore filter (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 PBS), 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 PBS), 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 (β-naphthyl phosphate disodium, fast blue BB salt, $MgSO_4$) for 5 min before being rinsed in water and dried. To confirm the position of the NS3 proteins, we autoradiographed the filter.

In vitro transcription and translation. The cDNA copy of BTV-10 S10 was recovered from the transfer vector pAcYM1.BTV.10.10 by *Bam*HI digestion and ligated into the *Bam*HI site in the polylinker of the dual promoter vector pSPT-18. 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. Transcripts were produced in a reaction mixture containing 400 μM of each nucleoside triphosphate, 1 μg of template DNA, 1 mM dithiothreitol, 40 mM Tris hydrochloride [pH 7.9], 6 mM $MgCl_2$, 2 mM spermidine, and 15 U of SP6 RNA polymerase. After 1 h at 37°C, 5 U of RNase-free DNase I were added and the incubation continued for a further 10 min. The RNA was recovered by phenol and then chloroform extraction and ethanol precipitated, and after confirmation on a 2% agarose gel, it was suspended in H_2O to give a concentration of approximately 0.5 μg/μl.

In vitro translations were performed with rabbit reticulocyte lysates (Amersham) containing 50 ng of RNA per μl and 0.5 μCi of [^{35}S]methionine (>800 Ci/mmol; Amersham; Dupont, NEN Research Products) per μl. After incubation at 30°C for 1 h, an equal volume of 2× dissociation buffer (see above) was added, the sample was heated to 100°C for 5 min, and the products were resolved on an 8 to 20% gradient PAGE gel.

BTV-10 dsRNA was prepared from infected BHK-21 cells as described by Yamaguchi et al. (26). Individual segments were separated on a 2% agarose gel, and the S10 segment was eluted. Prior to in vitro translation, the dsRNA was denatured with 10 mM methylmercuric hydroxide (17).

Peptide mapping of viral proteins. The procedure adopted was based on that of Cleveland and associates (1). After separation of the [^{35}S]methionine-labeled proteins on an 8 to 20% gradient Laemmli gel, the gel was dried and autoradiographed and the appropriate proteins were excised. The gel fragments were rehydrated in equilibration buffer (0.156 mM Tris hydrochloride [pH 6.8], 0.1% SDS), and gel slices containing similar levels of radioactivity were inserted into the wells of a 15 to 20% Laemmli gel cast with a deep (5-cm) stacking gel. The samples were overlaid with 20% glycerol in equilibration buffer followed by 10 μl of 10% glycerol in equilibration buffer containing *Staphylococcus aureus* V8 protease (250 ng) and bromophenol blue (0.001%). Electrophoresis was commenced immediately at 150 V, and when the tracking dye had traversed two-thirds of the stacking gel, the power was switched off for 30 min to allow digestion. After this period, the power was reconnected and electrophoresis continued until the tracking dye had moved to the bottom of the resolving gel. The peptides were identified by autoradiography after fixation and drying of the gel.

Preparation of antibodies in mice to expressed NS3. *S. frugiperda* cells were infected with the recombinant baculovirus and harvested at 48 h postinfection. Approximately 50 μg of expressed NS3 was resolved by gradient SDS-PAGE (10 to 20%) and located by KCl precipitation of the SDS-protein complexes. The gel slice was excised and crushed by successive passages through syringe needles of increasing gauges. A small volume of PBS was added, and the resulting slurry was injected intraperitoneally into two mice on day 0. Further injections of antigen were similarly administered on days 7, 14, and 21. An intraperitoneal injection of 2×10^6 Ehrlich ascites cells was given on day 18, and ascitic fluids were removed at intervals from days 22 to 30. After selection of the highest-titered samples, the ascitic fluid was cleaned by absorption with *S. frugiperda* cells (acetone powder).

Immunoprecipitation analysis. ^{35}S-labeled BTV-10 proteins in RIPA buffer from BHK-infected cells or from in vitro translation of BTV-10 dsRNA were incubated at 37°C with mouse anti-NS3 ascitic fluid for 2 h. Protein A-Sepharose CL-4B beads (in RIPA buffer) were then added, and the incubation continued for a further 2 h at room temperature with regular mixing before centrifugation and thorough washing of the beads. The precipitated proteins were removed by boiling for 5 min in dissociation buffer, resolved on a 10% SDS-PAGE gel, and located by fluorometry.

RESULTS

Construction of recombinant viruses. An essentially complete DNA copy of RNA segment S10 was inserted into the transfer vector pAcYM1 as described in Materials and Methods. Sequence analysis (Fig. 1) showed that during removal of the dG-dC tail, 10 nucleotides were also removed from the 5′ end of the cDNA. The initiation codon, however, remained intact. A similar number of nucleotides were also lost from the 3′ end (data not shown). The deleted 3′ residues were beyond the TGA termination codon of the open reading frame (residues 709 to 711 [13]).

The S10 gene was inserted into the AcNPV genome by cotransfecting *S. frugiperda* cells with pAcYM1.BTV-10.10 DNA and infectious wild-type AcNPV DNA. Plaques exhibiting a polyhedrin-negative phenotype were selected, and after further plaque purification, viral DNA was prepared. The presence of the S10 gene in the recombinant virus (designated AcYM1.BTV-10.10) was demonstrated by Southern analysis following *Bam*HI digestion of viral DNA (Fig. 2a). As expected, the nick-translated BTV-10.10 probe did not hybridize to AcNPV DNA but did hybridize to pAcYM1.BTV-10.10 (also *Bam*HI cut) and BTV-10.10 DNA. This latter sample, which acted as a further control and size marker, was prepared by *Pst*I digestion of the pBR322.BTV-10.10 clone followed by electroelution of the S10 insert. The fainter bands seen in the figure represent incomplete *Bam*HI digestion products.

Transcription of the S10 coding sequence in *S. frugiperda* cells was assessed by Northern analyses. Polyadenylated

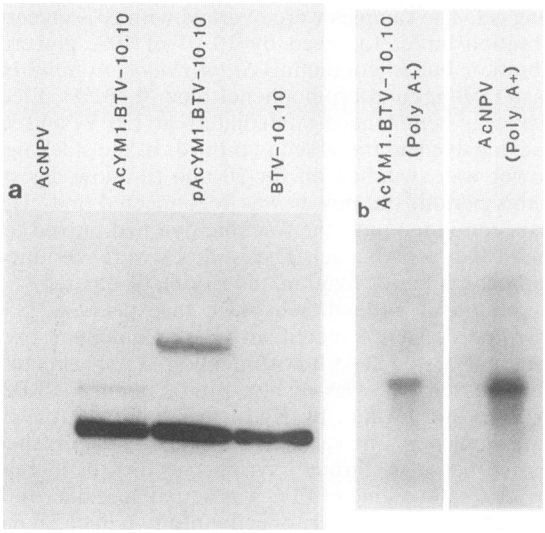

FIG. 2. Southern and Northern blot analysis of recombinant baculovirus nucleic acids. (a) *Bam*HI digests of DNA recovered from transfer vector pAcYM1.BTV-10.10, wild type AcNPV, and recombinant virus AcYM1.BTV-10.10 were resolved by agarose gel electrophoresis and probed with nick-translated BTV-10 S10 DNA. BTV-10.10 DNA from the original pBR322.BTV-10.10 clone was included as a marker and control. (b) Total cellular RNA was extracted from *S. frugiperda* cells infected with the recombinant baculovirus AcYM1.BTV-10.10 or wild type AcNPV. Polyadenylated species were selected, resolved on a denaturing methylmercuric hydroxide gel, and, after blotting to Hybond-N membrane, hybridized to nick-translated DNA probes as described in Materials and Methods. For the recombinant viral RNA, the probe consisted of BTV-10 S10 DNA; for the AcNPV RNA, a 550-base-pair fragment of the polyhedrin gene was used.

RNA species isolated from cells infected with the recombinant virus or wild-type AcNPV were hybridized to nick-translated probes representing the BTV-10 S10 DNA or a 545-base-pair fragment of the AcNPV polyhedrin gene as described in Materials and Methods. An mRNA band representing the polyhedrin transcript (approximately 1.1 kilobases) was identified by the 545-base-pair polyhedrin probe (Fig. 2b). Transcription of the BTV gene was indicated by hybridization of the BTV-10 S10 probe to a polyadenylated RNA species extracted from cells infected with the recombinant virus. As expected, the BTV mRNA species exhibited a migration similar to that of AcNPV polyhedrin mRNA since both genes are approximately the same size.

Expression of BTV-10 NS3 protein in *S. frugiperda* cells. Confluent monolayers of *S. frugiperda* cells were infected at a multiplicity of 10 PFU per cell and harvested at 24 or 48 h after infection. Proteins were resolved by SDS-PAGE and identified by staining with Coomassie brilliant blue R or by fluorography for cells pulse-labeled with [^{35}S]methionine prior to harvesting (Fig. 3).

In comparison with the 33-kDa polyhedrin protein induced by AcNPV (6), the recombinant virus synthesized two unique protein species (the putative NS3 and NS3A polypeptides) with approximate sizes of 25 and 24 kDa. Synthesis of the putative NS3A polypeptide was substantially less than that of the putative NS3 polypeptide and was only clearly observed after pulse-labeling. For comparison, ^{35}S-labeled BTV-10 proteins from BTV-10-infected BHK cells were included (Fig. 3). To estimate the approximate amounts of the proteins synthesized by the recombinant virus, we electrophoresed different concentrations (0.5 to 5 μg) of bovine serum albumin in parallel with the extracts of cells infected with the recombinant virus and scanned the protein

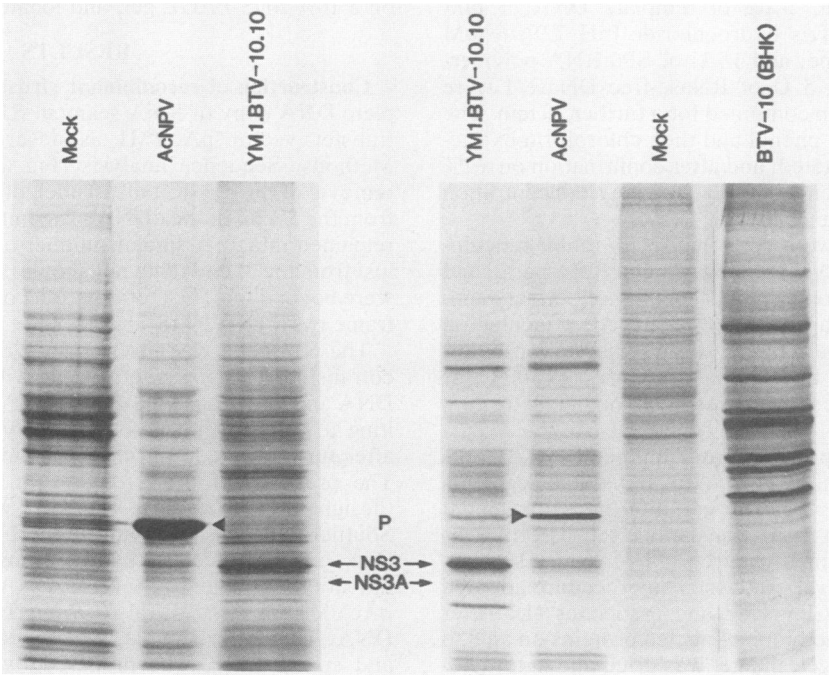

FIG. 3. Expression of NS3 by recombinant baculovirus. *S. frugiperda* cells were infected at a multiplicity of 10 PFU per cell with recombinant virus AcYM1.BTV-10.10 or wild-type AcNPV or were mock infected. Cells were harvested at 24 or 48 h postinfection, and after separation by SDS-PAGE, proteins were located either with Coomassie brilliant blue R (left side) or by fluorography for cells pulse-labeled with [^{35}S]methionine (right side). BTV proteins from pulse-labeled BTV-10-infected BHK cells are included for comparison. Details are given in Materials and Methods. The arrowheads denote bands that represent polyhedrin protein (P).

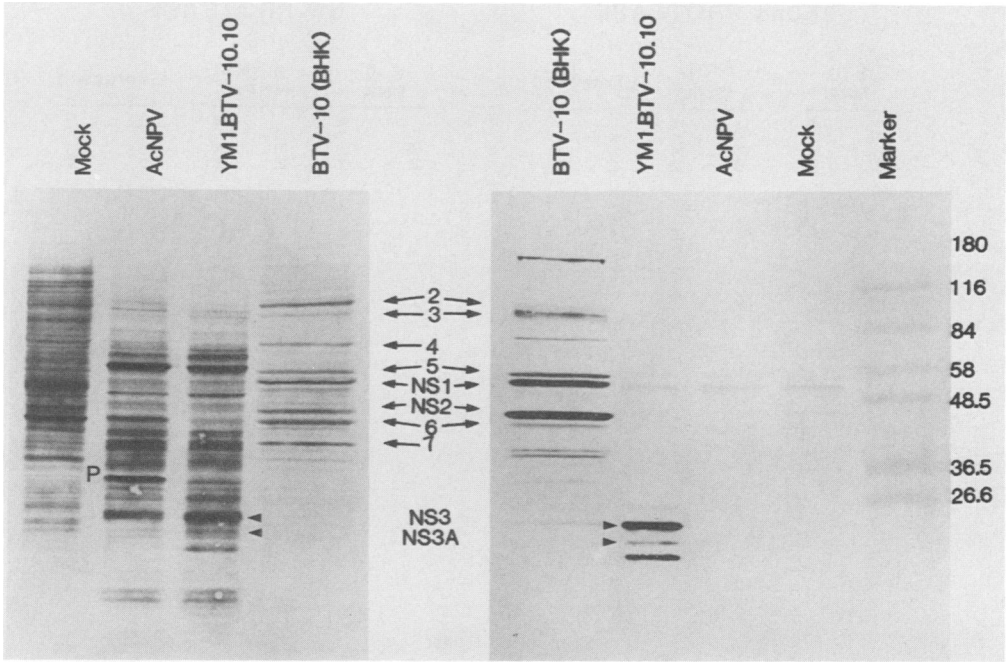

FIG. 4. Western blot analysis of proteins expressed by the recombinant baculovirus. ^{35}S-labeled proteins from BTV-10-infected BHK cells and *S. frugiperda* cells infected with AcNPV or the recombinant baculovirus were separated by SDS-PAGE and electroblotted onto Durapore membrane. The membrane was agitated in the presence of rabbit anti-BTV-10 serum for 90 min and then washed. After the membrane was agitated in conjugate for a further 90 min and rewashed, immune reactions were detected by the addition of substrate as described in Materials and Methods. Samples on the right side represent the blotted proteins, and those on the left side represent the autoradiograph of the blot. Numbers on right show size in kilodaltons.

bands in a Joyce-Loeb autodensitometer. By this means, the putative NS3 protein was estimated to be expressed at approximately 10 μg/10^6 cells.

Western blot analysis. Samples of ^{35}S-labeled proteins from *S. frugiperda* cells infected with the recombinant baculovirus or with wild-type AcNPV or from BHK-21 cells infected with BTV-10 were separated by gradient PAGE. The polypeptides were electroblotted onto a Durapore filter, and their reaction to rabbit anti-BTV-10 serum was assessed (Fig. 4). The NS3 protein synthesized by the recombinant baculovirus reacted strongly with the antiserum, while NS3A, which was present in a far lower amount, exhibited a correspondingly decreased reaction. It was concluded, therefore, that the expressed proteins represented the NS3 polypeptides. A third protein band that migrated ahead of NS3A also reacted with the antiserum and was assumed to result from degradation of the S10 gene products. In the control sample of BTV proteins from BTV-10-infected BHK cells, the antiserum reacted with VP2, -3, -5, -6, and -7 as well as with NS1 and NS2. No clear reaction was observed with the S10 gene products, although a polypeptide with an electrophoretic mobility similar to that of the expressed NS3 showed a slight reaction.

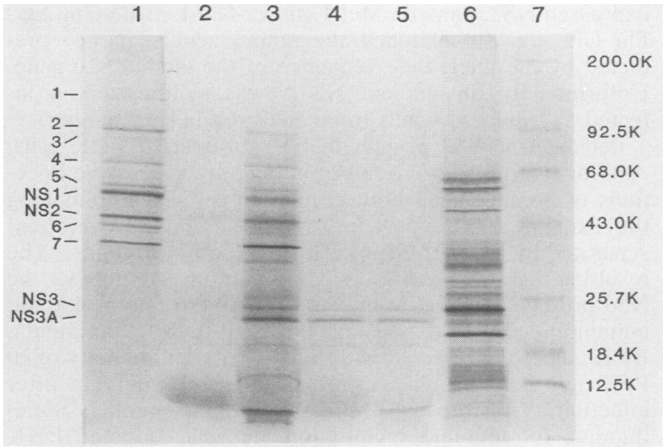

FIG. 5. PAGE analyses of in vitro translation products. The translation products of denatured BTV-10 total dsRNA (lane 3), S10 dsRNA (lane 4), and mRNA transcripts of the cDNA copy of BTV-10 S10 synthesized with SP6 RNA polymerase (lane 5) and with no exogenous RNA (lane 2) were analyzed by PAGE. For controls, methionine-labeled proteins from BHK-21 cells infected with BTV-10 (lane 1) and *S. frugiperda* cells infected with the recombinant baculovirus (lane 6) were also electrophoresed with the translated products. Numbers on right show size in kilodaltons.

In vitro translation and peptide mapping. To confirm that the proteins expressed by the recombinant baculovirus were BTV gene products and also to determine whether the expressed NS3 and NS3A proteins were related, we used peptide map analysis. As a control, dsRNA purified from BTV-10-infected BHK cells (both total RNA and S10 RNA isolated by agarose gel electrophoresis) was denatured with methylmercuric hydroxide and translated in the rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. The in vitro translation products of the total genomic BTV-10 RNA (Fig. 5, lane 3) contained proteins that corresponded to the BTV proteins synthesized in virus-infected BHK cells (Fig. 5, lane 1). The S10 products, which are known to be minor components in BTV-infected cells, could not be clearly distinguished in the cell extracts, although they were readily identified among the in vitro-translated products.

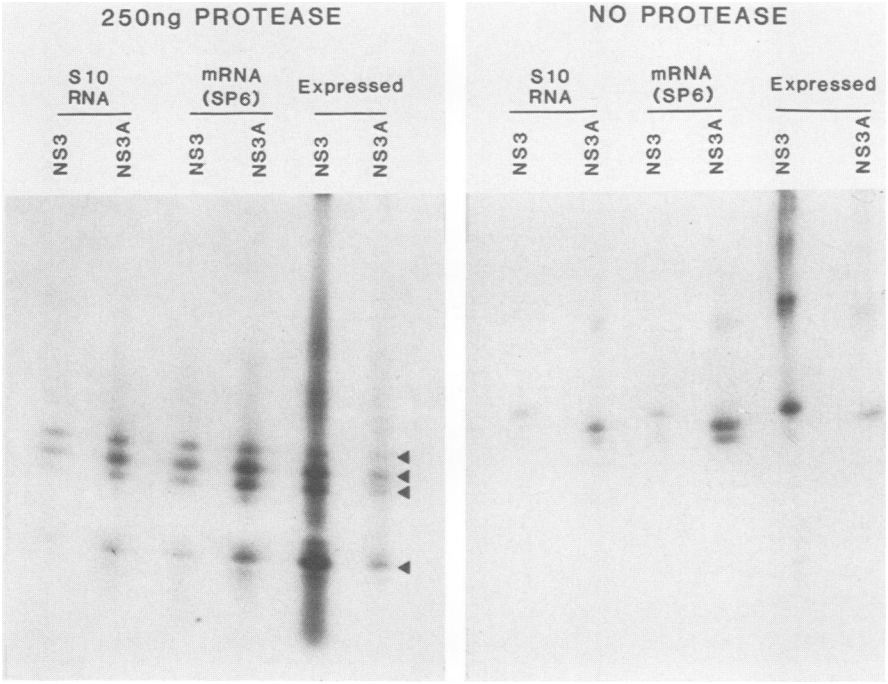

FIG. 6. Peptide mapping of NS3 and NS3A proteins by limited proteolysis. ^{35}S-labeled proteins from *S. frugiperda* cells infected with the recombinant baculovirus and from in vitro translation of denatured S10 dsRNA and mRNA from the cDNA copy were resolved by SDS-PAGE and located by fluorography. Bands representing NS3 and NS3A were excised, and segments were placed into the wells of a second SDS-15% polyacrylamide gel. These were overlaid with buffer containing 0 ng (control) or 250 ng of *S. aureus* V8 protease, and after limited proteolysis in the stacking gel, digestion products were separated in the resolving gel (1). After the gels were dried, peptides were located by fluorography. Arrowheads denote common digestion fragments.

The relative proportions in which the S10 gene products were synthesized were clearly different between the baculovirus-expressed material, in which NS3 was the predominant product, and the in vitro translation products of the dsRNA in the cell-free system, in which, as observed by others (17, 24), NS3 and NS3A were synthesized in approximately equimolar amounts (compare Fig. 4 and 5).

To assess whether removal of the 5′ (or 3′) noncoding nucleotides during the DNA manipulations affected the baculovirus expression of the gene, the cDNA copy from the transfer vector pAcYM1.BTV-10.10 was recloned into the dual promoter vector pSPT-18 and RNA transcripts were synthesized with SP6 RNA polymerase. When translated in the rabbit reticulocyte lysate system, these transcripts produced a pattern of NS3 and NS3A identical to that obtained with the denatured S10 dsRNA (Fig. 5, lanes 4 and 5), confirming the identity of the products made both by the recombinant baculovirus and by in vitro translation of denatured viral RNA.

For peptide analyses, the products from the in vitro translation of BTV-10 S10 RNA and that obtained from mRNA transcripts from the S10 cDNA together with ^{35}S-labeled recombinant baculovirus-expressed proteins were separated by PAGE, and the gel was dried. The NS3 and NS3A bands were located by autoradiography, excised, and, after rehydration, subjected to limited proteolysis with *S. aureus* V8 protease (see Fig. 6). A similar profile of peptides was seen in all samples, indicating that both BTV NS3 and NS3A proteins were synthesized in the insect cells infected with the recombinant virus. Also, the similarity between the peptide maps of NS3 and NS3A reported by others (20, 24) was confirmed.

Amino acid sequencing of expressed NS3. NS3 protein synthesized in *S. frugiperda* cells by the recombinant baculovirus was separated by SDS-PAGE and electroblotted onto Immobilon membrane. After staining with Coomassie brilliant blue R, the NS3 band was excised and the N terminus of the protein was sequenced in an Applied Biosystem 470 sequenator. The sequence obtained for the expressed NS3, namely, Met-Leu-Ser-Gly-Leu-Ile-Gln-Agr-Phe-Glu, exactly matched the amino acid sequence predicted by the nucleotide sequence of the cloned S10 gene. Unfortunately, insufficient NS3A was synthesized in infected *S. frugiperda* cells to be analyzed in this manner.

Detection of NS3 protein in BTV-10-infected BHK cells. Antibodies to NS3 were raised in mice by successive injections of homogenized polyacrylamide gel slices containing the expressed NS3 protein (but not the NS3A protein) separated by SDS-PAGE (see Materials and Methods). The resulting high-titer ascitic fluid was then used to detect the NS3 proteins in BTV-10-infected cells by Western blot and immunoprecipitation analysis. For both these experiments, BHK cells were infected with BTV-10 at a multiplicity of 10 PFU per cell and harvested at different intervals after infection. Viral proteins were pulse-labeled with [^{35}S]methionine for immunoprecipitation analysis. Infected BHK cell lysates and purified baculovirus NS3 protein were subjected to SDS-PAGE, electroblotted, and allowed to react with anti-NS3 ascitic fluid in Western blot analysis (Fig. 7a). Alternatively, the BHK cell extracts or in vitro-translated products of viral RNA were directly mixed with a sample of the ascitic fluid, and immune complexes were recovered by precipitation with Sepharose CL-4B (Fig. 7b). In both studies, NS3 and NS3A were clearly observed in the

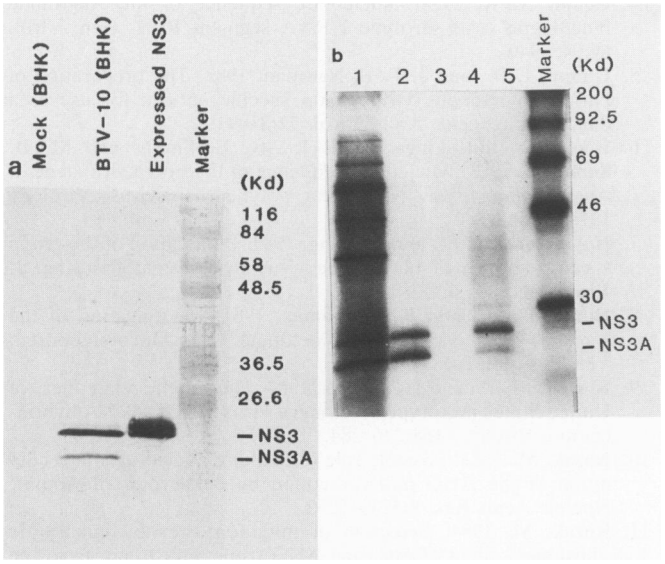

FIG. 7. Western blot and immunoprecipitation analysis of NS3 and NS3A from BTV-10-infected BHK cells. BHK cells were infected with BTV-10 at a multiplicity of 10 PFU per cell and harvested at 18 h postinfection. (a) Proteins were separated by SDS-PAGE (15%) and electroblotted onto Immobilon membrane, and the S10 gene products were detected by reaction with mouse anti-NS3 ascitic fluid. Purified NS3 protein from *S. frugiperda* cells infected with the recombinant baculovirus acted as a control and marker. Kd, Kilodaltons. (b) Immunocomplexes formed by the addition of mouse ascitic fluid were recovered following precipitation with Sepharose CL-4B and analyzed by SDS-PAGE. Denatured total BTV-10 dsRNA translated in the rabbit reticulocyte lysate system was similarly subjected to immunoprecipitation with mouse ascitic fluid. Shown are the in vitro-translated products of BTV-10 dsRNA (lane 1) immunoprecipitated with anti-NS3 ascitic fluid (lane 2) or control ascitic fluid (lane 3). BTV-10-infected BHK cell lysates were similarly immunoprecipitated with anti-NS3 ascitic fluid (lane 4) or control ascitic fluid (lane 5).

cell extracts at 18 h postinfection, with NS3 being present in greater quantity. The ability of the ascitic fluid to react with both NS3 and NS3A with approximately the same affinity was confirmed by the precipitation of both proteins in equal amounts after in vitro translation of BTV-10 dsRNA (Fig. 7b).

Purified BTV-10 particles were also subjected to Western blot analysis with the anti-NS3 ascitic fluid. Although this experiment was repeated several times with large amounts of concentrated virus, no NS3 proteins were identified in the virus preparation (data not shown).

Western blot analysis of expressed NS3 with sheep anti-BTV antiserum. RNA-RNA hybridization studies have shown that the S10 gene is highly conserved within the North American BTV isolates (9). Gould (4) has recently cloned and sequenced the S10 gene of Australian BTV serotype 1. Comparison of this sequence with that of serotype 10 revealed only 14 amino acid differences, half of which were conserved changes. Such data further suggest that the NS3 gene is highly conserved. To examine the immunogenic relatedness of NS3 among homologous and heterologous BTV serotypes, we investigated the reactivity of sheep anti-BTV sera (to serotypes 10, 11, 13, and 17) by Western blot analysis. All four anti-BTV antisera tested reacted strongly with the baculovirus-expressed NS3 and NS3A of serotype 10 (Fig. 8). No reactivity with control sheep antiserum was observed.

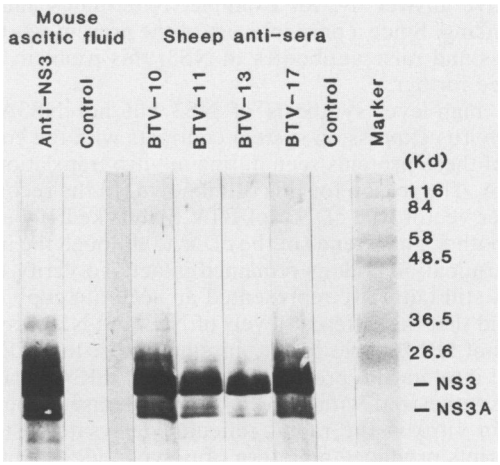

FIG. 8. Western blot analysis of expressed NS3 and NS3A with sheep anti-BTV antisera. *S. frugiperda* cells were infected with the recombinant baculovirus and harvested at 24 h postinfection. Proteins were resolved by SDS-PAGE (15%), electroblotted, and reacted with polyclonal anti-BTV-10, -BTV-11, -BTV-13, -BTV-17, or control sheep antisera. Mouse ascitic fluid (control or anti-NS3) was similarly reacted. Kd, Kilodaltons.

DISCUSSION

RNA segment 10 of BTV has been demonstrated in vitro to code for two gene products, NS3 and NS3A (17, 24). These proteins have an almost identical peptide map, suggesting that NS3A represents an alternative or derived form of NS3. Following the discovery of two in-phase AUG codons at bases 20 to 22 and 59 to 61 in S10 of BTV-10 (13) and BTV-1 (4), it has been suggested that NS3 and NS3A arise from independent translation initiation at these sites. No data have been reported to support this hypothesis or the alternative that processing of the complete gene product is involved. The low concentration of the NS3 proteins in vivo has been a major obstacle in resolving the origin of NS3A and also in ascribing a function to the proteins. To investigate these issues, we constructed a baculovirus expression vector containing a cDNA copy of the BTV-10 S10 gene and isolated a recombinant virus. *S. frugiperda* cells infected with the recombinant baculovirus synthesized a major product (NS3) of approximately 25 kDa. This was shown to be the BTV S10 product by Western blot analysis with anti-BTV-10 sera and by peptide mapping with the in vitro-translated products of BTV-10 S10 dsRNA as a control. N-terminus amino acid sequencing provided the final confirmation, as well as the proof that for NS3, translation is initiated from the first AUG codon.

A second polypeptide slightly smaller than NS3 (24 kDa) was also identified in insect cells infected with the recombinant baculovirus. This was designated NS3A based on its reaction with BTV-10 antiserum, its peptide map profile, and the fact that its migration was the same as that of NS3A made by in vitro translation of S10 dsRNA. The Western blot analyses, however, identified other products that migrated ahead of the NS3 proteins. Unless these represent aborted translation products, it is possible that they represent degradation derivatives of the NS3 proteins. This was not investigated further. Whether the observed NS3A protein is the result of independent initiation of translation or is a degraded derivative of NS3 (either in vitro or in vivo) is not known. Unfortunately, the expressed NS3A is present in such low levels that it has not been possible to obtain a

definitive answer by, for example, N-terminus amino acid sequencing. Since a principle aim of the present work was to express and raise antibodies to NS3, this problem was not pursued further.

The high-level synthesis of NS3 but not NS3A in the baculovirus expression system contrasts with the equimolar ratio of these proteins seen during in vitro translation of S10 dsRNA. The reason for this is unknown. In the recombinant transfer vector, the cDNA of BTV S10 lacked 10 base pairs from both 5′ and 3′ ends of the cDNA, although the initiation and termination codons remained intact. To verify that this cDNA still faithfully represented an accurate copy of RNA S10 and that the different levels of NS3 and NS3A synthesis were not attributable to the missing bases, the cDNA was ligated into an SP6 promoter vector and mRNA transcripts were synthesized with SP6 RNA polymerase. When translated in vitro in the rabbit reticulocyte lysate system, the transcripts produced a pattern of polypeptides identifical to those obtained with the purified denatured S10 dsRNA.

Antibodies raised in mice to the expressed NS3 were used to investigate the presence of the S10 gene products in BTV-10-infected BHK cells. Both NS3 and NS3A were detected late in the infection cycle (18 h) by immunoprecipitation and Western blot analysis. Whether they are synthesized at an earlier time but are not present in sufficient quantities to detect is unknown. The relative abundance of NS3 compared with NS3A in vivo was analogous to that seen during expression of the S10 gene in the baculovirus system. This difference in the proportions of the S10 gene products between in vivo and in vitro systems is particularly noteworthy. It indicates that erroneous deductions may result when using rabbit reticulocyte lysate to predict the pattern of protein synthesis from a gene containing alternative start codons.

The high-level expression of NS3 by the recombinant baculovirus provided sufficient material for functional analysis. This is currently in progress. The absence, however, of NS3 or NS3A in purified BTV-10 particles (as determined by Western blot analysis) excludes the suggestion that these proteins are structural elements in the outer capsid. The positive reaction of sera from sheep infected with different BTV serotypes with the expressed NS3 of BTV-10 reveals it to be a group-specific antigen whose function would seem to depend on a high degree of conservation.

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