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PHYLOGENETIC STUDIES OF THE UNITED STATES BLUETONGUE VIRUSES AND CHARACTERIZATION OF THE VIRAL VP4 PROTEIN

by

I-Jen Huang

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology
(Molecular Biology)

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1996

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ABSTRACT

Phylogenetic Studies of the United States Bluetongue Viruses and Characterization of the Viral VP4 Protein

by

I-Jen Huang, Doctor of Philosophy

Utah State University, 1996

Major Professor: Dr. Joseph K.-K. Li
Department: Biology (Molecular Biology)

Bluetongue virus (BTV) is transmitted by arthropod vectors and causes bluetongue disease with serious economic loss in many regions of the world. The replication mechanism of bluetongue virus is still not clear. To have a better understanding regarding the viral replication, the function of each individual protein has to be identified. This study used molecular biology techniques to investigate the function of the inner core protein VP4.

The M1 genes of United States bluetongue virus serotypes-2, -10, -11, -13, and -17 were cloned and sequenced. The length of each of the five M1 genes is 1981 nucleotides. The coding region of the M1 gene, which encodes the VP4 protein, possesses an open reading frame with an

initiation codon (ATG) at nucleotides #9-11 and a stop codon (TAA) at nucleotides #1941-1943. This open reading frame encodes a protein of 644 amino acid residues with a predicted molecular weight of about 75 kDa. A potential leucine zipper motif was detected near the carboxyl terminus of the deduced VP4 amino acid sequence. The phylogenetic analysis of bluetongue viruses using the sequences of these five cognate M1 genes is consistent with the results of previous phylogenetic studies. Serotypes-10, -11, -13, and -17 are closely related and serotype-2 is the most distantly related among the five US BTV serotypes.

Heterologously expressed bluetongue virus VP4 protein was purified to near homogeneity. Six linear epitopes of VP4 were mapped at both termini and in the middle of the protein. By using enzyme-linked immunosorbent assay and peptide competition assay, six linear epitopes were found to be surface accessible. The VP4 protein was shown to be an oligomer by chemical cross-linking. VP4 protein was identified as a ssRNA-binding protein. The VP4 protein has binding activity towards both capped and non-capped ssRNA. RNA-binding activity was not specific to BTV ssRNA. A leucine-zipper motif of VP4 is not required for RNA-binding activity. One RNA-binding domain was mapped between amino acid residues #112-158 by a Northwestern assay and by

deletion mutant analysis. Using sequence-specific synthetic peptides corresponding to VP4 in the arginine- and lysine-rich regions, four potential ssRNA-binding domains of VP4 protein were mapped.

(190 pages)

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CHAPTER I

LITERATURE REVIEW AND PROJECT OUTLINE

INTRODUCTION

Bluetongue viruses

In the early twentieth century, Spreull (1905) described an animal disease that he believed to be found in South Africa only. The infected sheep had high fever lasting for 5 to 7 days followed by conspicuous lesions that emerged in the mouth. The tongue was severely affected and became dark blue. Spreull proposed "bluetongue" as the name of this disease. This disease was later found in both domestic animals (sheep, goat, cattle, etc.) and in wild ruminants (blesbuck, elk, etc.) in most parts of the world. Bluetongue disease is an infectious, noncontagious, arthropod-borne viral disease, mostly of sheep (Erasmus, 1975). The disease can occur, clinically, as an acute, subacute, mild, or inapparent disease. In domestic sheep and white-tailed deer (*Odocoileus virginianus*), clinical signs occur rapidly within a short viral incubation. Goats, cattle, elk (*Cervus elaphus canadensis*), and other wild ruminants develop subacute or inapparent infections and are considered to have a longer viremia (Sohn and Yuill, 1991). The first sign of the

bluetongue disease is that the infected animal's body temperature rises with an average duration of 5 to 7 days, which may be transitory or continue for up to 14 days. In most of the cases, hyperemia and swelling of the buccal and nasal mucosa are observed at the time of temperature rise. The mouth area will have lesions resulting in excoriation, leaving bleeding, ulcerated lesions, which become infected and often necrotic. During this time the infected sheep develops lethargy and anorexia. Death may result from lesions of the smooth muscle of the esophagus and pharyngeal area and from heart failure (Marht and Osburn, 1986b). Abortion, fetal death, and congenital malformations are also associated with bluetongue disease (Luedke *et al.*, 1977; Osburn *et al.*, 1971). The potential danger of this disease was intensively discussed in an international symposium on bluetongue in 1974. Geering (1975) suggested that bluetongue disease was in an epizootic situation and could cause a mortality rate up to 70% in a highly susceptible sheep population.

The bluetongue disease is caused by bluetongue virus (BTV) classified in the *Orbivirus* genus of the family *Reoviridae* (Borden *et al.*, 1971; Murphy *et al.*, 1971). The viruses are transmitted among animals by insect vectors such as the biting midges of the species *Culicoides* (Wirth

and Dyce, 1985) and distributed to most parts of the world. *Orbivirus* is designated for a group of viruses that are spread by arthropods and have similar morphological characteristics (Borden *et al.*, 1971). The word "orbi" means circle or ring, referring to the round-shaped surface of this group of viruses. This group of viruses shares similar morphological and physicochemical features within the family *Reoviridae,* but there is not a common antigen to all viruses in the *Orbivirus* genus (Borden *et al.*, 1971; Verwoerd *et al.*, 1979). There are 14 serogroups in this genus distinguished by complement fixation tests (Borden *et al.*, 1971; Karabatsos, 1985), including African horse sickness, bluetongue, changuinola, corriparta, epizootic hemorrhagic disease, equine encephalosis, eubenangee, lebombo, orungo, palyam, umatilla, wallal, warrego, and kemerovo. Based on neutralization tests, there are 24 serotypes of BTV identified around the world, and only five serotypes (BTV-2, -10, -11, -13, and -17) are found in United States (US) (Barber, 1979; Mecham *et al.*, 1986; Roy, 1989).

Bluetongue virus is an icosahedral-shaped particle consisting of a double-layered protein coat and has a 10-segmented double-stranded RNA (dsRNA) genome (Verwoerd *et al.*, 1972). The double-layer viral protein coat contains

two outer capsid proteins (VP2 and VP5) and five inner core proteins (VP1, VP3, VP4, VP6, and VP7). Similar to rotavirus and reovirus, BTV has concentric protein shells and has no envelope. The negatively stained BTV virion has a fuzzy appearance and a diameter of about 70 nm (Verwoerd *et al.*, 1972; Martin and Zweerink, 1972). It does not have any discernible structure, although virus-derived cores have icosahedral symmetry. Using cryoelectron micrographic image analyses, the outer capsid has revealed a well-ordered morphology, and it is different from those of human rotavirus and reovirus (Prasad *et al.*, 1988; Yeager *et al.*, 1990; Metcalf *et al.*, 1991). From these images the proteins of the outer capsid have two shapes; one is globular and the other is sail-shaped (Hewat *et al.*, 1992). The sail-shaped spikes are predicted to be the haemagglutinating protein VP2, which contains the virus-neutralizing epitope. The globular-shaped protein is more likely to be VP5. The structure of the inner core has been examined by cryoelectron microscopy to a resolution of 30 Å. The inner core has a diameter of 69 nm, and it contains 260 copies of VP7 trimers and 120 copies of VP3 (Burroughs *et al.*, 1995). The crystal structure of the inner core was recently grown up to 0.8 mm in diameter. The dimensions are 754 x 796 x 823 $Å^3$ (Burroughs *et al.*, 1995). The second

layer of the inner core contains the remaining three minor proteins (VP1, VP4, and VP6) and the genomic dsRNA. A loss of infectivity is found after one or two of the outer capsid proteins are removed. Both divalent and monovalent cations are able to destabilize the outer capsid layer (Huismans *et al.*, 1987c). The inner core particles are stable at a pH as low as 5, and the virions are the most stable at the pH range of 8 to 9. BTV virions are also stable in nonionic detergent and in lipid solvents (Huismans *et al.*, 1987c; Mertens *et al.*, 1987; Verwoerd *et al.*, 1979).

Verwoerd and his colleagues (1970) separated the genome of BTV into 10 segments using polyacrylamide gel electrophoresis. The electrophoretic pattern of the genomic RNA segments is quite different from that of reovirus, and the molecular weight of the BTV genome is estimated to be 19×10^6 Da (Fukusho *et al.*, 1989).

Most dsRNA segments of the five US BTV serotypes have been cloned and sequenced recently. The length of segment ranges from 3944 to 822 base pairs (bp). The lengths of the corresponding dsRNA segments of each serotype of BTV are identical or very similar, and their sequences of cognate genes are all highly conserved. A sequence of six nucleotides at the 5′-and 3′- end of all BTV dsRNA segments

is conserved; however, the significance of this sequence conservation is still unknown. The migration profiles of the genomic dsRNA segments on a polyacrylamide gel are different for each BTV serotype. This characteristic can be used to distinguish different serotypes of BTV diagnostically.

Viral proteins

Each of the 10 segments of the viral genome except the S4 gene codes for one polypeptide (Grubman *et al.*, 1983; Sanger and Mertens, 1983; Roy, 1989; Evans-Wade *et al.*, 1992). The double-layer viral protein coat contains two outer capsid proteins (VP2 and VP5) and five inner core proteins (VP1, VP3, VP4, VP6, and VP7). Four nonstructural proteins (NS1, NS2, NS3, and NS3A) have also been detected in BTV-infected cells (Hyatt *et al.*, 1991) and by *in vitro* translation (Grubman *et al.*, 1983; Mertens *et al.*, 1984; Van Dijk and Huismans, 1988). The viral structural proteins from purified virions can be resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) into distinctive patterns for each serotype. Thus, each serotype of BTV can be distinguished by comparing the electrophorogram patterns in addition to the neutralization

assay. The following are the known characteristics of these viral proteins.

VP1 protein

VP1, the largest protein of BTV, is one of the minor inner core proteins and is coded by the L1 gene. Only six copies of VP1 are potentially present in each virion (Huismans and Van Dijk, 1990). The L1 gene has 3944 nucleotides (Huang *et al.*, 1995) and is highly conserved among five US BTVs. VP1 is a highly basic protein with a positively charged carboxyl terminus. The molecular weight of VP1 is estimated to be 150 kDa from the deduced amino acid sequences (Huang *et al.*, 1995).

The deduced amino acid sequence of VP1 has about 30% identity with those of some of the prokaryotic and eukaryotic polymerases such as the beta-chain subunits of *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* RNA polymerase, respectively (Roy *et al.*, 1988). By using the cell lysate of *Spodoptera frugiperda* (SF) cells infected with a recombinant baculovirus expressing the VP1 protein, labeled ATP was incorporated into the poly(U)-templates, indicating that VP1 is a potential BTV RNA polymerase (Urakawa *et al.*, 1989). However, there is no report of using purified BTV VP1 for the polymerase activity assay,

yet. Recently, four of the linear epitopes were mapped at amino acid residues #42-55, #459-473, #881-903, and #1251-1274 (Huang *et al.*, 1995) using oligoclonal antibodies produced in rabbits.

VP2 protein

VP2 protein is a major component of the outer capsid of BTV. It is the serotype-specific antigen, and can elicit neutralizing antibodies (Huismans and Erasmus, 1981; Kahlon *et al.*, 1983). It is also the protein responsible for the hemagglutination of red blood cells (Cowley and Gorman, 1987). The segment coding for VP2 is the L2 gene (Verwoerd *et al.*, 1972). The lengths of L2 genes of the five US BTVs are slightly different; 2943 bp in BTV-2 (Yamaguchi *et al.*, 1988), 2923 bp in BTV-17, 2935 bp in BTV-13, and 2926 bp in both BTV-10 and -11 (Purdy *et al.*, 1985). The molecular weight of VP2 is about 111 kDa, and there are 180 copies of VP2 per virion, calculated by cryoelectron microscopy (Hewat *et al.*, 1992). The deduced amino acid sequences of VP2 vary considerably among all serotypes. Peptide mapping is further evidence that VP2 is unique for each of the US serotypes (Mecham *et al.*, 1986). However, each has a highly conserved carboxyl terminus, middle region between amino acid #360-367 (NPYPCLRG), and

six conserved cysteines. These conserved regions may be responsible for VP2 maintaining its overall structure or may be required for interactions with other viral proteins. The residue variability for cognate VP2 reflects the role of this protein in serotype-specific neutralizing antibody production. VP2 purified from SDS-PAGE was not able to elicit neutralizing antibodies. However, recombinant VP2 expressed in insect cells was able to produce neutralizing antibodies (Inumaru and Roy, 1987), indicating that the neutralizing epitopes could be conformational. Hwang and Li (1993) have mapped some of the potential neutralization epitopes of BTV-2 (amino acid residues #397-408 and #428-442), BTV-10 (amino acid residues #199-213 and #248-265), BTV-11 (amino acid residues #82-94 and #310-321), and BTV 17 (amino acid residues #31-43 and #444-464).

VP3 protein

VP3 protein is one of the major inner core proteins. It is encoded by the L3 gene (2772 bp in length) and is highly conserved among serotypes (Hwang *et al.*, 1994). It has been shown to contain a serogroup-specific antigen (Huismans and Erasmus, 1981), similar to VP7. From the analysis of the deduced amino acid sequences, VP3 has a low content of charged amino acids and a high content of hydrophobic amino acids (Hwang *et al.*, 1994). The

molecular weight of VP3 is about 103 kDa with a pI of 6 (Hwang *et al.*, 1994), and there are 120 copies of VP3 per virion (Burroughs *et al.*, 1995). It exists as a dimer that forms a scaffold upon which the trimers of VP7 reside. When VP3 and VP7 (the two major inner core proteins) were co-expressed in a baculovirus expression system, the formation of a viral core-like particle (CLP) was found (French and Roy, 1990). It was concluded that the assembly of the inner core does not require the presence of other viral proteins nor the whole viral genome (French and Roy, 1990). The methionine at residue #500 and the arginine at residue #502 of VP3 were found to be required for the CLP formation (Tanaka and Roy, 1994). The CLP formed by VP3 and VP7 was shown to bind BTV and non-BTV single-stranded RNA (ssRNA) and also DNA. The binding activity was shown to reside on VP3 (Loudon and Roy, 1992).

VP4 protein

VP4 is one of the minor inner core proteins. An estimated 5 to 10 molecules are present per virion (Huismans and Van Dijk, 1990). The molecular weight of this protein is about 75 kDa. The segment encoding VP4 is the M1 gene. It has the shortest 5'-noncoding region among all BTV genes. Hybridization studies indicated that the sequences among BTV are highly conserved. VP4 of BTV-10

has a high content of charged amino acids (Yu *et al.*, 1987), which is common in many of the nucleic acid binding proteins. The function of VP4 is still unknown. In a recent study (Le Blois *et al.*, 1992), VP4 has exhibited GTP-binding activity in both purified virions and cell lysate containing recombinant VP4 expressed in a baculovirus expression system. The 5′ ends of the mRNAs of BTV are believed to be capped and methylated during transcription, like reovirus and rotavirus; however, it has never been experimentally demonstrated. Due to the GTP-binding activity of VP4, it is the prime candidate for the guanylyltransferase of BTV.

VP5 Protein

The segment coding for VP5 protein is the M3 gene which has 1638 bp, with an open reading frame that can code for 526 amino acids. The molecular weight of this protein is about 59 kDa (Purdy *et al.*, 1986; Hirasawa and Roy, 1990; Oldfield *et al.*, 1991; Yang and Li, 1992). Along with VP2, VP5 is another major outer capsid protein of BTV. VP5 is the only structural protein that is glycosylated (Yang and Li, 1993). It was shown that VP5 can bind to different types of lectins including *Galanthus nivalis* agglutinin (GNA), *Sambucus nigra* agglutinin (SNA), Peanut

agglutinin (PNA), and *Datura stramonium* agglutinin (DSA) but not *Maackia amurensis* agglutinin (MAA).

The functions of VP5 have not been determined. From the deduced amino acid sequence analyses of the M3 gene, we know that VP5 is very variable (Yang and Li, 1992), but not as variable as the VP2 protein. VP5 is enriched with non-polar amino acids (such as isoleucine and alanine). Unlike the VP2 protein, the VP5 protein cannot elicit neutralization antibodies against BTV (Marshall and Roy, 1990) even though it is located in the outer capsid. However, Huismans *et al.* (1983) showed that a mixture of VP2 and VP5 induced a higher titer of the neutralizing antibodies than VP2 alone.

Two antigenic epitopes of VP5 were mapped (Yang *et al.*, 1992b). One linear epitope was identified between amino acid residues #175-189. This epitope is common and conserved in all five US BTV serotypes. The other epitope was found to be conformational and located between amino acid residues #33 to #215.

VP6 protein

VP6 is one of the minor inner core proteins with a molecular weight of 35 kDa (Roy *et al.*, 1990; Hwang *et al.*, 1992b). It is encoded by the S3 gene, which is 1049 bp in

length (Fukusho *et al.*, 1989; Hwang *et al.*, 1992b). There are approximately 37 copies of VP6 per virion (Huismans and Van Dijk, 1990). The sequences of S3 genes among BTVs are highly conserved (Hwang *et al.*, 1992b). A single, long ORF of the S3 gene is identified in all five US BTVs (Hwang *et al.*, 1992b). This ORF encodes a 325-amino acid VP6 of BTV-10, -11, -13, and -17. However, the ORF coding for BTV-2 VP6 is only 301 amino acids in length, which is 24 amino acids shorter than others. This is due to a shift of an initiation codon from nucleotides #28-30 to #100-102. Sequence analyses of VP6 have revealed a common motif for a helicase. The helicase activity may be involved in unwinding the dsRNA genome for the mRNA synthesis. However, no such activity has yet been found and reported.

Hayama and Li (1994) mapped six linear antigenic epitopes of VP6 at amino acid residues #35-46, #63-78, #181-193, #200-209, #216-227, and #292-302. Four additional conformational epitopes were mapped recently (Hayama and Li, manuscript in preparation). These epitopes are conserved among all five US BTVs. By using recombinant VP6 expressed in *E.coli*, Hayama and Li (1994 and manuscript in preparation) have identified the nucleic acid binding activity and domains of VP6. It has been shown that VP6 binds to ssRNA, ssDNA, dsRNA, and dsDNA. Two domains

responsible for dsDNA and dsRNA-binding are mapped at amino acid residues #181-209 and #292-302 near the carboxyl terminus. Domains for the ssRNA- and ssDNA-binding were also mapped at the same two locations (Hayama and Li, manuscript in preparation).

VP7 protein

VP7 is the major inner core protein, with a molecular weight of 38 kDa. The protein comprises 36% of the total core proteins (Huismans *et al.*, 1987c). It has serogroup-specific antigenic determinants (Huismans and Erasmus, 1981; Gumm and Newman, 1982). The segment coding for VP7 is the S1 gene, which is 1156 bp in length. The S1 gene has an ORF capable of coding for 349 amino acid residues. The sequences of S1 genes and the deduced amino acid sequence for each VP7 are highly conserved among all five US BTVs. Only a single lysine residue is located at amino acid residue #255 in VP7, determined from the deduced amino acid sequence. By using endoproteinase lys-C to digest all five US BTVs VP7 proteins, the presence of this single lysine residue of VP7 was confirmed (Li and Yang, 1990). VP7 is very hydrophobic, reflecting its location and interior preference. However, Hyatt and Eaton (1988) and Lewis and Grubman (1990) showed that monoclonal antibodies against VP7 reacted with intact virions, and concluded that

a portion of the VP7 extrudes to the surface of the BTV virion. Similar results were obtained by immunoelectron microscopy (Eaton *et al.*, 1991). VP7 was clearly shown to be myristylated when BTV was purified from infected BHK-21 cells containing ^{3}H-myristic acids in the medium (Huang and Li, unpublished observation). It seems that VP7 is the only major myristylated structural protein of BTV. The significance of this posttranslational modification is still unknown.

Five linear antigenic epitopes were mapped at amino acid residues #1-60, #207-240, #256-275, #339-349, and #122-139 (Li and Yang, 1990; Wang *et al.*, 1996). These epitopes were highly conserved among the VP7 proteins of all five US BTVs. In addition to the linear epitopes, one conformational and potential serogroup-specific immunodominant epitope was identified at the N-terminus of VP7 proteins between amino acid residue #30 and 48 (Wang *et al.*, 1994).

VP7 proteins exist in the inner core as trimers (780 copies per virion). Recently, VP7 was crystallized and analyzed at 2.6 Å resolution. In this study it was shown that VP7 consists of two domains, one a ß-sandwich, the other a bundle of α-helices, and a short carboxy-terminal arm (Grimes *et al.*, 1995). The carboxy-terminal arm is

predicted to tie the trimers together during capsid formation. A new study has shown that VP7 proteins are orientated with their N-terminus accessible on the surface of the intact virion by immunoelectron microscopy (Wang *et al.*, 1996), although VP7 is one of the major inner core proteins.

NS1 protein

There are four nonstructural proteins found in BTV-infected cells. NS1 and NS2 are the two major non-structural proteins, whereas NS3 and NS3A are barely detectable. NS1 protein is synthesized in large amounts and represents about 25% of the total virus-specified protein in infected cells. It forms high molecular weight tubular structures (Huismans, 1979; Huismans and Els, 1979). These tubules are formed in the cytoplasm of BTV-infected cells as early as 4 hr postinfection (Huismans, 1979) and before the formation of the significant amounts of virus inclusion bodies (VIBs) and viral progeny (Hyatt *et al.*, 1989). From electron microscopic studies of BTV-infected cells it was evident that these tubules accumulate late in infection, particularly in peri-orjuxtonuclear locations. The segment coding for NS1 is the M2 gene, which is 1769 bp in length (Lee and Roy, 1987; Grubman and Samal, 1989; Wang *et al.*,

1989; Hwang *et al.*, 1993). This gene has an ORF with a coding capacity of 552 amino acids (64 kDa). From sequence analyses, the nucleotide sequence identity of M2 genes among five US BTVs is between 92 to 99%; the deduced amino acid sequence identity of NS1 is between 96-99% (Hwang *et al.*, 1993). NS1 is rich in cysteine residues, and all 16 cysteines are conserved in the cognate NS1 proteins of different BTV serotypes. This indicates the probability that NS1 may have highly ordered disulfide bonds.

Two cysteines (amino acid residues #337 and 340) located in a hydrophilic region of NS1 were found to be required for tubule polymerization (Monastyrskaya *et al.*, 1994). In addition to these two cysteines, the first 10 amino acids in the amino-terminus and the 20 amino acids in the carboxy-terminus are also required for the tubule polymerization in insect cells that were infected by baculovirus expressing recombinant NS1 (Monastyrskaya *et al.*, 1994). The mutated NS1 proteins in the infected insect cells made ribbon-like structures instead of tubular polymers.

Eaton *et al.* (1987) reported that NS1 was detected in cytoskeleton-associated virus particles, purified virions, and core particles; and suggested that viral morphogenesis

of BTV may proceed in this tubular structure composed of NS1.

NS2 Protein

NS2 protein is the nonstructural protein that forms VIBs in the cytoplasm of BTV-infected cells. It is the only BTV protein that is phosphorylated (Huismans *et al.*, 1987b; Devaney *et al.*, 1988). The segment that encodes for NS2 is the S2 gene, which contains 1125 nucleotides (Yang *et al.*, 1992a) with an ORF coding for 354 amino acid residues. The molecular weight of NS2 is about 41 kDa from the deduced amino acid sequence. The protein is hydrophilic and rich in charged amino acids. Two of the linear antigenic epitopes were mapped at amino acid residues #153-166 (DIRELRQKIKNERE) and #274-286 (EKVAKQIKLKDER) (Huang and Li, unpublished observation). NS2 from BTV-infected cells (Huismans *et al.*, 1987b), recombinant NS2 expressed in insect cells (Thomas *et al.*, 1990), and recombinant NS2 expressed in *E. coli* (Li *et al.*, unpublished observation) has the ability to bind BTV mRNA and non-BTV ssRNA but not dsRNA. The potential function of NS2 is the selection and condensation of the BTV mRNAs during viral morphogenesis.

The ssRNA-binding activity of the NS2 protein has been intensively studied. Recently, the ssRNA-binding domains of the NS2 protein were identified at the amino terminus (Zhao *et al.*, 1994). Point-mutational studies indicated that the arginines at amino acid residues #6 and #7 and the lysine at residue #4, but not the glutamic acid at residue #2, are involved in ssRNA-binding. However, in our laboratory, there are another three potential ssRNA-binding domains that have been mapped at amino acid residues #2-11, #153-166, and #274-286 (unpublished observation). By deletion it has been shown that neither the amino- nor carboxy-terminus of NS2 is involved in VIB formation (Zhao *et al.*, 1994).

NS3/3A Protein

The other nonstructural proteins of BTV are NS3 and NS3A. Both proteins are synthesized in small amounts and at the late infection stage. Both of them are encoded by the smallest segment of BTV dsRNA, the S4 gene (Mertens *et al.*, 1984; Van Dijk and Huismans, 1988). The S4 gene is 822 bp in length and has two long, in-phrase open reading frames (Hwang *et al.*, 1992a).

The *in vitro* translation products of S4 mRNA contain two proteins, NS3 and NS3A, with a molecular weight of 28

and 25 kDa, respectively. The two proteins are produced by the two in-phase ORFs of the S4 gene. The two proteins have almost identical peptide maps and are synthesized in very small amounts in BTV-infected cells (Van Dijk and Huismans, 1988). After the first AUG was deleted, only NS3A was synthesized but NS3 was not synthesized (Wu *et al.*, 1992). From the deduced amino acid sequences of NS3, at least two conserved hydrophobic domains at amino acid residues #118-147 and #156-182 among all five US BTVs may represent transmembrane domains. Hyatt *et al.* (1991), using immunoelectron microscopic studies, reported that NS3 and NS3A are associated with intra-cellular, smooth-surfaced vesicles, and with the plasma membrane.

In a recent study by Wu *et al.* (1992), NS3 and NS3A were identified as N-linked glycoproteins containing high-mannose sugars such as polylactosaminoglycans. In this study it was also suggested that the carbohydrate chains are not required for the NS3 to be transported to the cell surface.

The function(s) of NS3 and NS3A are still unknown. A hypothesis is that they are involved in the release of BTV from infected cells during the final stages of BTV morphogenesis.

Viral replication

BTV replicates in the cytoplasm of infected cells and infection finally leads to cell death. The mechanism of BTV replication is still largely unknown. BTV is taken up by endocytosis after attachment to cells (Lecatsas, 1968; Cromack *et al.*, 1971). The binding of BTV to cells is mediated by the outer capsid protein VP2 (Huismans *et al.*, 1983). However, the viral receptor has not been identified. The virus adsorbs to susceptible cells within 20 min at both 4^0 C and 37^0 C (Huismans *et al.*, 1983). This suggested that the membrane fluidity of the infected hosts is not involved in the entry of BTV. The outer coat protein VP2 is then removed in endosomes within 1 hr of infection (Huismans *et al.*, 1987c). VP5 is probably removed at the same location, also prior to release of the core particle into the cytoplasm. The release of virus from endosomes is dependent on the low pH within this organelle. Increasing the pH of endosomes by adding NH_4Cl and methylamine (weak bases) inhibited the entry of endocytosed virus particles into the cytoplasm (Ohkuma and Poole, 1978). In the cytoplasm, core particles of BTV bind to intermediate filaments (Eaton *et al.*, 1987; Eaton and Hyatt, 1989) and transcribe virion mRNAs (Bowne and Jochim, 1967). The transcriptase activity of virions requires the

removal of both outer capsid proteins (VP2 and VP5) (Van Dijk and Huismans, 1980). The mRNA species are synthesized at different rates (Huismans and Verwoerd, 1973). The NS1 mRNA seems to be the most abundant in the early stage of infection. Huismans *et al.* (1987c) suggested that the transcriptase activity of the core particle may be active for a limited time, and unlike reovirus, core particles do not reassociate with newly synthesized outer coat proteins to release from cells as progeny virus. Translation then occurs, using the newly transcribed mRNAs. The generated viral proteins appear to condense with single strand-RNAs (ssRNAs) to form core-like particles, which ultimately aggregate to generate viral inclusion bodies (VIBs). The new viral core particles are made within the VIB matrix (Hyatt *et al.*, 1989). Complementary RNAs will then be synthesized using the plus-sense RNAs as templates to complete the formation of dsRNAs. By studying the size and density of the particles, it has been suggested that the particles leaving the VIB contain dsRNAs; therefore, BTV replicase may be found within the VIB. A portion of the outer coat proteins is added to the core particles and the remainder may follow when the virus leaves the VIBs. The morphogenesis of BTV occurs within the VIBs, and a high

proportion of viral progeny remains associated with cell debris (Howell and Verwoerd, 1971).

Diagnosis of bluetongue disease

The clinical diagnosis of bluetongue disease in sheep is uncomplicated for an experienced clinician (Gibbs, 1983). The diagnosis of this disease in cattle is often clinically unnoticeable, and it is very easily confused with other diseases such as malignant cattarhal fever and bovine virus diarrhea/mucosal disease. Isolation of BTV from infected animals is difficult for diagnosis. Detection of antibodies to BTV is based on group-reactive tests (including complement-fixation, ELISA, fluorescent-antibody, *etc.*) and the serotype-specific neutralization tests (Pearson *et al.*, 1985). In a recent report it was indicated that competitive enzyme-linked immunosorbent (C-ELISA) can be used as a rapid and specific test for serodiagnosis of BTV infection in llamas and other wild ruminants (Afshar *et al.*, 1995).

The most common antigens used for detecting BTV antibodies in infected animals are NS1 (Urakawa and Roy, 1988; He *et al.*, 1991) and VP7 (Lunt *et al.*, 1988). In the study done by He *et al.* (1991), sheep anti-NS1 antibodies were raised with the recombinant NS1 expressed in *E. coli*

and used to detect the NS1 proteins of the five US BTVs. The immunological activity of the antibody was indistinguishable between native and recombinant NS1. This indicated that NS1 can be used as a group-specific antigen in the diagnosis of BTV infections, in addition to VP7.

New techniques have been developed for the detection of BTV in biological specimens. These methods include polymerase chain reaction (PCR) and nucleic acid hybridization (Wade-Evans *et al.*, 1990; Venter *et al.*, 1991). Wade-Evans *et al.* (1990) used a group of primers designed complementary to the termini of the 10 dsRNAs of South Africa BTV serotype 1 (BTV-1SA) to amplify each individual segment. They found that the S1 gene gave the best amplification results. This primer was able to amplify the S1 gene from red blood cells (RBC) and buffy coat samples from cattle which were infected with BTV-4. Venter *et al.* (1991) also determined the genomic probes of BTV that can give the best sensitivity to detect BTV-specified RNA in infected cells. They found that the probe derived from the genome segment that encodes NS1 (M2 gene) to be the most sensitive. It could detect BTV-specified RNA in cells as early as two to three hr postinfection, and it could also be used to analyze the cells infected at very low multiplicities of infection (M.O.I.). The great

sensitivity of this probe is due to M2 mRNA, the target of the probe, which is transcribed more frequently than other viral mRNAs. Similar types of experiments were also shown in epizootic hemorrhage disease virus (EHDV) and encephalitis virus (EEV).

Vaccine development

Development of a vaccine against BTV took place as early as 1908 (Theiler, 1908). This was accomplished by immunizing sheep with an attenuated strain of BTV that had been serially passaged in sheep. Chemically inactivated viruses were also used for the vaccination. Unfortunately, these have been shown unsafe and unsuccessful (Neitz, 1948; Mahrt and Osburn, 1986a; Mahrt and Osburn, 1986b) due to the capability of the viruses to reassort in mixed infections.

Huismans *et al.* (1987a) had shown that purified VP2 alone could elicit neutralizing antibodies against BTV and protect the animal challenged with the same serotype BTV. Inumaru and Roy (1987) and Marshall and Roy (1990) used recombinant BTV neutralizing antigen(s) expressed in insect cells to produce neutralizing antibodies against BTV. However, these types of approaches are expensive and only protect the homologous but not heterologous BTV serotypes. Another approach has been investigated using synthetic

peptides as subunit vaccine. Synthetic peptide vaccine has the advantages of being inexpensive, stable, and can produce high titer antibodies. If a common neutralization epitope of BTV serotypes is identified, it can be used as a subunit vaccine to protect animals from all BTV serotypes.

JUSTIFICATION AND OBJECTIVES

Unlike reovirus, the replication of BTV is still not clear. Many of the assumptions regarding mRNA synthesis and genomic RNA replication are based on information obtained from reovirus research. After the outer coat proteins are removed from the virion, the transcriptase is activated and transcribes mRNAs using the dsRNA genome segments as templates (Van Dijk and Huismans, 1980; Martin and Zweerink, 1972). The mRNAs are capped and translated to viral proteins. The mRNAs are also used as templates for synthesizing negative-sense RNAs to form the dsRNA genome. All 10 segments of dsRNA are always sorted in a single virion. These characteristics of BTV transcription and replication are considered to be the functions of the inner core minor proteins. However, the functions of the inner core proteins are still unknown. To understand these phenomena, each of the inner core proteins has to be characterized, and their functions have to be identified.

VP1 expressed as a recombinant protein in the baculovirus expression system has a potential RNA polymerase activity. It might be an RNA-dependent RNA polymerase of BTV (Urakawa *et al.* 1989). VP6 recently has been characterized as a nucleic acid binding protein (Hayama and Li, 1994 and manuscript in preparation). It has a nonselective nucleic acid binding activity *in vitro*. VP4, a minor component in the inner core of BTV, is encoded by the M1 gene. Huismans (1979) suggested that only 5 to 10 molecules of VP4 are present in each virion. Very little information is known about the function(s) and characteristics of VP4. The only information known regarding the function of VP4 is that both viral and recombinant VP4 expressed in a baculovirus expression system have GTP-binding activity (Le Blois *et al.*, 1992). VP4 is potentially a guanylyltransferase of BTV.

The objective of my study is to investigate the function of VP4. I will determine the nucleic acid sequence of the M1 gene that codes for VP4. Based on the deduced amino acid sequence, I plan to locate some of the potential functional motifs of VP4 by comparing its amino acid sequence with other known sequences and to perform structural and functional analysis of these domains. This information will reveal the potential role(s) of VP4 in

virus replication and morphogenesis. I will also compare the VP4 sequences of five US BTV serotypes to identify the conserved and probably essential domains of VP4 which may be required for its function.

In our laboratory we have determined the genetic relationships among BTV serotypes (Kowalik and Li, 1989; Kowalik *et al.*, 1990; Hwang *et al.*, 1992a; Hwang *et al.*, 1992b; Yang and Li, 1992; Yang *et al.*, 1992a; Hwang *et al.*, 1993; Hwang *et al.*, 1994; Huang *et al.*, 1995). Sequencing the M1 genes of all five US BTV serotypes will produce further information for phylogenetic analysis of the US BTV serotypes.

Because only a small amount of VP4 is present in the virus, it is very difficult, costly, and time-consuming to obtain this protein from the purified virus and elucidate its potential function(s). Recombinant VP4 will be used in this study.

The specific aims of this study are:

(1) To clone and determine the nucleotide sequences of the M1 gene that codes for VP4 of five US BTV serotypes.

(2) To compare the phylogeny of five US BTV serotypes by analyzing their five M1 gene sequences.

(3) To express and purify recombinant VP4.

(4) To map and characterize some of the functional domains of VP4.

REFERENCES

Afshar, A., Heckert, R.A., Dulac, G.C., Trotter, H.C., and Myers, D.J. (1995). Application of a competitive ELISA for the detection of bluetongue virus antibodies in llamas and wild ruminants. J. Wildlife Disease **31**, 327-330.

Barber, T.L. (1979). Temporal appearance, geographic distribution, and species of origin of bluetongue serogroups in the United States. Am. J. Vet. Res. **40**, 1654-1656.

Borden, E.C., Shope, R.E., and Murphy, F.A. (1971). Physiochemical and morphological relationship of some arthropod-borne viruses to bluetongue virus--a new taxonomic group: Physiochemical and serological studies. J. Gen. Virol. **13**, 261-271.

Bowne, J.G., and Jochim, M.M. (1967). Cytopathologic change and development of inclusion bodies in cultured cells infected with bluetongue virus. Am. J. Vet. Res. **28**, 1091-1105.

Burroughs, J.N., Grimes, J.M., Mertens, P.P.C., and Stuart, D.I. (1995). Crystallization and preliminary X-ray analysis of the core particle of bluetongue virus. Virology, **210**, 217-220.

Cowley, J.A., and Gorman, B.M. (1987). Genetic reassortments for identification of the genome segment coding for the bluetongue virus haemagglutinin. J Virol. **61**, 2304-2306.

Cromack, A.S., Blue, J.L., and Gratzek, J.B. (1971). A quantitative ultrastructural study of the development of bluetongue virus in Madin-Darby bovine kidney cells. J. Gen. Virol. **13**, 229-244.

Devaney, M.A., Kendall, J., and Grubman, M.J. (1988). Characterization of a non-structural phosphoprotein of two orbiviruses. Virus Res. **11**, 51-164.

Eaton, B.T., Gould, A.R., Hyatt, A.D., Coupar, B.E.H., Martyn, J.C., and White, J.R. (1991). A bluetongue serogroup-reactive epitope in the amino terminal half of the major core protein VP7 is accessible of the surface of bluetongue virus particles. Virology **180**, 687-696.

Eaton, B.T., and Hyatt, A.D. (1989). Association of bluetongue virus with the cytoskeleton. Subcell Biochem. **15**, 229-269.

Eaton, B.T., Hyatt, A.D., and White, J.R. (1987). Association of bluetongue virus with the cytoskeleton. Virology **157**, 107-116.

Erasmus, B.J. (1975). Bluetongue in sheep and goats. Aust. Vet. J. **51**, 165-170.

Evans-Wade, A.M., Mertens, P.P.C., and Belsham, G.J. (1992). Sequence of genome segment 9 of bluetongue virus (serotype 1, South Africa) and expression analysis demonstrating that different forms of VP6 are derived from initiation of protein synthesis at two distinct sites. J. Gen. Virol. **73**, 3023-3026.

French, T.J., and Roy, P. (1990). Synthesis of empty BTV core particles by a recombinant baculovirus expressing the two major structural core proteins. J. Virol. **64**, 1530-1536.

Fukusho, A., Yu, Y., Yamaguchi, Y., and Roy, P. (1989). Completion of the sequence of bluetongue virus serotype 10 by the characterization of a structural protein, VP6, and a non-structure protein, NS2. J. Gen. Virol. **70**, 1677-1689.

Geering, W.A. (1975). Control of bluetongue in an epizootic situation: Australian plans. Aust. Vet. J. **51**, 220-224.

Gibbs, E.P.J. (1983). Bluetongue disease. Agripractice **4**, 13.

Grimes J., Basak A.K., Roy P., and Stuart D. (1995). The crystal structure of bluetongue virus VP7. Nature **373**, 167-170.

Grubman, M.J., Appleton, J.A., and Letchworth, G.J. (1983). Identification of bluetongue virus serotype 17 genome segments coding for polypeptides associated with virus

neutralization and intergroup reactivity. Virology **131**, 353-366.

Grubman, M.J., and Samal, S.K. (1989). Nucleotide and deduced amino acid sequence of the nonstructural protein NS1, of the US bluetongue virus serotype 17. Nucleic Acids Res. **17**, 10498.

Gumm, I.D., and Newman, J.F.E. (1982). The preparation of purified bluetongue virus group antigen for use as a diagnostic reagent. Arch. Virol. **72**, 83-93.

Hayama, E., and Li, J.K.-K. (1994). Mapping and characterization of antigenic epitopes and the nucleic acid binding domains of the VP6 protein of bluetongue viruses. J. Virol. **68**, 3604-3611.

He, X.S., Wang, L.F., Doi, R.H., Maid, M., Osburn, B.I., and Chuang, R.Y. (1991). Expression of a full-length nonstructural protein NS1 of bluetongue virus serotype 17 in *Escherichia Coli*. Biochem. Biophys. Res. Commun. **180**, 994-1001.

Hewat, E.A., Booth, T.F., Wade, R.H., and Roy, P. (1992). Structure of bluetongue virus particles by cryo-electron microscopy. J. Struct. Biol. **109**, 1-69.

Hirasawa, T., and Roy, P. (1990). The complete nucleotide sequence of VP5 of a strain of bluetongue virus of serotype 2 isolated in the USA reveals is close relation with a virus of serotype 1 isolated in Australia. Virus Res. **15**, 107-112.

Howell, P.G., and Verwoerd, D.W. (1971). Bluetongue virus. In: African swine fever virus and bluetongue virus (W.R. Hess, P.G. Howell, and D.W. Verwoerd , eds.), pp. 35-74. Springer, Berlin.

Huang, I-J., Hwang, G.-Y., Yang, Y.-Y., Hayama, E., and Li, J.K.-K. (1995). Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five US bluetongue viruses. Virology **214**, 280-288.

Huismans, H. (1979). Protein synthesis in bluetongue virus-infected cells. Virology **92**, 385-396.

Huismans, H., and Els, H.J. (1979). Characterization of the microtubules associated with the replication of three different orbiviruses. Virology **92**, 397-405.

Huismans, H., and Erasmus, B.J. (1981). Identification of the serotype-specific and serogroup-specific antigens of bluetongue virus. Onderstepoort J. Vet. Res. **48**, 51-58.

Huismans, H., Van der Walt, N.T., Cloete, M., and Erasmus, B.J. (1983). The biochemical and immunological characterization of bluetongue virus outer capsid polypeptides. In: Double-stranded RNA viruses (R.W. Compans and D.H.L. Bishop, eds.), pp. 165-172. Elsevier, New York.

Huismans, H., Van der Walt, N.T., Cloete, M., and Erasmus, B.J. (1987a). Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. Virology **157**, 172-179.

Huismans, H., and Van Dijk, A.A. (1990). Bluetongue virus structural components. Curr. Top. Microbiol. Immunol. **162**, 21-37.

Huismans, H., Van Dijk, A.A., and Bauskin, A.R. (1987b). *In vitro* phosphorylation and purification of a nonstructural protein of bluetongue virus with affinity for single-stranded RNA. J. Virol. **61**, 3589-3595.

Huismans, H., Van Dijk, A.A., and Els, H.J. (1987c). Uncoding of parental bluetongue virus to core and subcore particles in infected L cells. Virology **157**, 180-188.

Huismans, H., and Verwoerd, D.W. (1973). Control of transcription during the expression of the bluetongue virus genome. Virology **52**, 81-88.

Hwang, G.-Y., Yang, Y.-Y., Chiou, J.-F., and Li, J.K.-K. (1992a). Sequence conservation among the cognate nonstructural NS3/3A protein genes of six bluetongue viruses. Virus Res. **23**, 151-161.

Hwang, G.-Y., Yang, Y.-Y., Chiou, J.-F., and Li, J.K.-K. (1992b). Comparative sequences analyses of the cognate structural protein VP6 genes of five US bluetongue viruses. Virus Res. **24**, 315-323.

Hwang, G.-Y., and Li, J.K.-K. (1993). Identification and localization of a serotypic neutralization determinant on the VP2 protein of bluetongue virus 13. Virology **195**, 859-862.

Hwang, G.-Y., Chiou, J.-F., Yang, Y.-Y., and Li, J.K.-K. (1993). High-sequence conservation among the United States bluetongue viruses cognate M2 genes which encode the nonstructural NS1 tubule protein. Virology **192**, 321-327.

Hwang, G.Y., Xiang, M., and Li, J.K.-K. (1994). Analyses and conservation of sequences among the cognate L3 segments of the five United States bluetongue viruses. Virus Res. **32**, 381-389.

Hyatt, A.D., and Eaton, B.T. (1988). Ultrastructural distribution of the major capsid proteins within bluetongue virus and infected cells. J. Gen. Virol. **69**, 805-815.

Hyatt, A.D., Eaton, B.T., and Brookes, S.B. (1989). The release of bluetongue virus from infected cells and their superinfection by progeny virus. Virology **173**, 21-34.

Hyatt, A.D., Gould, A.R., Coupar, B., and Eaton, B.T. (1991). Localization of the non-structural protein NS3 in bluetongue virus-infected cells. J. Gen. Viol. **72**, 2263-2267.

Inumaru, S., and Roy, P. (1987). Production and characterization of neutralization antigen VP2 of bluetongue virus serotype 10 using a baculovirus expression vector. Virology **157**, 472-479.

Kahlon, D.L., Sugiyama, K. and Roy, P. (1983). Molecular bases of bluetongue virus neutralization. J. Virol. **48**, 627-632.

Karabatsos, N. (1985). International catalogue of orbiviruses including certain other viruses of vertebrates, 3rd ed. The American Society of Tropic Medicine and Hygiene, San Antonio, Texas.

Kowalik, T.F., and Li, J.K.-K. (1989). Sequence analyses and structural predictions of double-stranded RNA segment S1 and VP7 from United States prototype

bluetongue virus serotypes 13 and 10. Virology **172**, 189-195.

Kowalik, T.F., Yang, Y.-Y., and Li, J.K.-K. (1990). Molecular cloning and comparative sequence analyses of bluetongue virus S1 segments by selective synthesis of specific full-length DNA copies of dsRNA genes. Virology **177**, 820-823.

Le Blois, H., French, T., Mertens, P.P.C., Burroughs, J.N., and Roy, P. (1992). The expressed VP4 protein of bluetongue virus binds GTP and is the candidate guanylyl transferase of virus. Virology **189**, 757-761.

Lecatsas, G. (1968). Electron microscopic study of the formation of bluetongue virus. Onderstepoort J. Vet. Res. **35**, 139-149.

Lee, J., and Roy, P. (1987). Complete sequence of the NS1 gene (M6 RNA) of US bluetongue virus serotype 10. Nucleic Acids Res. **15**, 7207.

Lewis, S.A., and Grubman, M.J. (1990). Bluetongue virus: Surface exposure of VP7. Virus Res. **16**,7-26.

Li, J.K.-K., and Yang, Y.-Y. (1990). Mapping to two immunodominant antigenic epitopes conserved among the major inner capsid protein, VP7 or five US bluetongue viruses. Virology **178**, 552-559.

Loudon, P.T., and Roy P. (1992). Interaction of nucleic acids with core-like and subcore-like particles of bluetongue virus. Virology **191**, 231-236.

Luedke, A.J., Jochim, M.M., and Jones, R.H. (1977). Bluetongue in cattle: Effects on calves previously infected in utero. Am. J. Vet. Res. **37**, 1701-1704.

Lunt, R.A., White, J.R., and Della-Porta, A.J. (1988). Studies with enzyme-linked immunosorbent assays for the serodiagnosis of bluetongue and epizootic haemorrhagic disease of deer. Vet. Microbiol. **16**, 323-338.

Mahrt, C.R., and Osburn, B.I. (1986a). Experimental bluetongue infection of sheep; effect of vaccination: Clinical and immunological studies. Am. J. Vet. Res. **47**, 1191-1197.

Mahrt, C.R., and Osburn, B.I. (1986b). Experimental bluetongue infection of sheep; effect of vaccination: Pathologic, immunofluorescent, and ultrastructural studies. Am. J. Vet. Res. **47**, 1198-1203.

Marshall, J.J.A., and Roy, P. (1990). High level expression of the two outer capsid proteins of bluetongue virus serotype 10: Their relationship with the neutralization of virus infection. Virus Res. **15**, 189-196.

Martin, S.A., and Zweerink, H.J. (1972). Isolation and characterization of two type of bluetongue virus particles. Virology **50**, 495-506.

Mecham, J.O., Dean, V.C., and Jochim, M.M. (1986). Correlation of serotype specificity and protein structure of the five US serotypes of bluetongue viruses. J. Gen. Virol. **67**, 2617-2624.

Mertens, P.P.C., Brown, F., and Sanger, D.V. (1984). Assignment of the genome segments of bluetongue virus type 1 to the proteins which they code. Virology **135**, 207-217.

Mertens, P.P.C., Burroughs, J.N., and Anderson, J. (1987). Purification and properties of virus particles, infectious subviral particles, and core of bluetongue virus serotype 1 and 4. Virology **156**, 375-386.

Metcalf, P.M., Cyrklaff, M., and Adrian, M. (1991). The three dimensional structure of reovirus obtained by cyro-electron microscopy. EMBO J. **10**, 3129-3136.

Monastyrskaya, K., Booth, T., Nel, L., and Roy, P. (1994). Mutation of two cysteine residues or deletion of the amino or carboxyl terminus of nonstructural protein NS1 of bluetongue virus abrogates virus-specified tubule formation in insect cells. J. Virol. **68**, 2169-2178.

Murphy, F.A., Borden, E.C., Shope, R.E., and Harrison, A. (1971). Physiochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus-a new taxonomic group: electron microscopic studies. J. Gen. Virol. **13**, 273-278.

Neitz, W.O. (1948). Immunological studies on bluetongue in sheep. Onderstepoort J. Vet. Sci. Anim. **23**, 93-136.

Ohkuma, S., and Poole, B. (1978). Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75**, 3327-3331.

Oldfield, E., Hirasawa, T., and Roy, P. (1991). Sequence conservation of the outer capsid protein: VP5, of bluetongue virus, a contrasting feature to the outer capsid protein VP2. J. Gen. Virol. **72**, 449-451.

Osburn, B.I., Silverstein, A.M., Prendergast, R.A., Johnson, R.T., and Parshall, C.J. (1971). Experimental viral-induced congenital encephalopathies. I. Pathology of hydroencephaly and porencephaly caused by bluetongue vaccine virus. Lab. Invest. **25**, 197-205.

Pearson, J.E., Carbrey, E.A., and Gustafson, G.A. (1985). Bluetongue and related orbivirus diagnosis in the United States. In: Bluetongue and related orbiviruses. (T.L. Barber, M.M. Jochim, and B.I. Osburn, eds.), pp. 469-475. Alan R. Liss, New York.

Prasad, B. V.V., Wang, G.J., Clerx, J.M.P., and Chiu, W. (1988). Three-dimensional structure of rotavirus. J. Mol. Biol. **199**, 269-275.

Purdy, M.A., Ghiasi, H., Rao, C.D., and Roy, P. (1985). Complete sequence of bluetongue virus L2 RNA that codes for the antigen recognized by neutralizing antibodies. J. Virol. **55**, 826-839.

Purdy, M.A., Ritter, G.D., and Roy, P.C. (1986). Nucleotide sequence of cDNA clones encoding the outer capsid protein, VP5, of bluetongue virus serotype 10. J. Gen. Virol. **67**, 957-962.

Roy, P. (1989). Bluetongue virus genetics and genome structure. Virus Res. **13**,179-206.

Roy, P., Fukusho, A., Ritter, D.G., and Lyons, D. (1988). Evidence for genetic relationship between RNA and DNA viruses from the sequence homology of a putative polymerase of bluetongue virus with that of vaccinia virus: Conservation of RNA polymerase genes from diverse species. Nucleic Acid Res. **16**, 11759-11767.

Roy, P., Marshall, J.A.A., and French, T.J. (1990). Structure of bluetongue virus genome and its encoded proteins. Curr. Top. Microbiol. Immunol. **162**, 43-87.

Sanger, D.V., and Mertens, M.J. (1983). Comparison of type 1 bluetongue virus protein synthesis *in vivo* and *in vitro*. In: Double-stranded RNA viruses (R.W. Compans and D.H.L. Bishop, eds), pp. 183-191. Elsevier, New York.

Sohn, R., and Yuill, T.M. (1991). Bluetongue and epizootic hemorrhagic disease in wild ruminants. Bulletin of the Society of Vector Ecology **16**, 17-24.

Spreull, J. (1905). Malarial catarrhal fever (bluetongue) of sheep in South Africa. J. Comp. Pathol. Ther. **18**, 321-337.

Tanaka, S., and Roy, P. (1994). Identification of domains in bluetongue virus VP3 molecules essential for the assembly of virus cores. J. Virol. **68**, 2795-2802.

Theiler, A. (1908). Inoculation of sheep against bluetongue and results in practice. Vet. J. **64**, 600-607.

Thomas, C.P., Booth T.F., and Roy, P. (1990). Synthesis of bluetongue virus encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells; it binds the single-stranded RNA species. J. Gen. Virol. **71**, 2073-2083.

Urakawa, T., and Roy, P. (1988). Bluetongue virus tubules made in insect cells by recombinant baculoviruses; expression of the NS1 gene of bluetongue virus serotype 10. J. Virol. **62**, 3917-3927.

Urakawa, T. Ritter, D.G., and Roy, P. (1989). Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: Association of VP1 with RNA polymerase activity. Nucleic Acids Res. **17**, 3919-3927.

Van Dijk, A.A., and Huismans, H. (1980). The *in vitro* activation and further characterization of the bluetongue virus-associated transcriptase. Virology **104**, 347-356.

Van Dijk, A.A., and Huismans, H. (1988). *In vitro* transcription and translation of bluetongue virus mRNA. J. Gen. Virol. **69**, 573-581.

Venter, E.H., Viljoen, G.J., Nel, L.H., Huismans, H., and Van Dijk, A.A. (1991). A comparison of different genomic probes in the detection of virus-specified RNA in orbivirus-infected cells. J. Virol. Methods **32**, 171-180.

Verwoerd, D.W., Louw, H., and Oellermann, R.A. (1970). Characterization of bluetongue virus ribonucleic acid. J. Virol. **5**, 1-7.

Verwoerd, D.W., Els, H.J., DeVilliers, E.M., and Huismans, H. (1972). Structure of the bluetongue virus capsid. J. Virol. **10**, 783-794.

Verwoerd, D.W., Huismans, H., and Erasmus, B.J. (1979). Orbiviruses. In: Comprehensive virology (H. Fraenkel-Conrad and R.R. Wagner eds.), Vol. **14**, pp. 285-345. Plenum Press, New York.

Wade-Evans, A.M., Mertens, P.P.C., and Bostock, C.J. (1990). Development of the polymerase chain reaction for the detection of bluetongue virus in tissue samples. J. Virol. Methods **30**, 15-24.

Wang, L.-F., Doi, R.H., Osburn, B.I., and Chang, R.Y. (1989). Complete sequence of the NS1 gene (S6 RNA) of US bluetongue virus serotype 17. Nucleic Acids Res. **17**,8002.

Wang, L.-F., Scanlon, D.B., Kattenbelt, J.A., Mecham, J.O., and Eaton, B.T. (1994). Fine mapping of a surface-accessible, immunodominant site on the bluetongue virus major core protein VP7. Virology **204**, 811-814.

Wang, L.-F., Hyatt, P.L., Andrew, M., Li, J.K.-K., and Eaton, B.T. (1996). Topography and immunogeneticity of bluetongue virus VP7 epitopes. Arch. Virol. **144**, 111-123.

Wirth, W.W., and Dyce. A.L. (1985). The current taxonomic status of the *Culicoides* vectors of bluetongue viruses. In: Bluetongue and related orbiviruses (T.L. Barker and M.M. Jochim, eds.), pp. 151-164. Alan R. Liss, New York.

Wu, X., Iwata, H., Chen, S.-Y., Compans, R.W., and Roy, P. (1992). Multiple glycoproteins synthesized by the

smallest RNA segment (S10 of bluetongue virus) J. Virol. **66**, 7104-7112.

Yamaguchi, S., Fukusho, A., and Roy, P. (1988). Complete sequence of neutralization protein VP2 of the recent US isolate bluetongue virus serotype 2: Its relationship with VP2 species of other US serotypes. Virus Res. **11**, 49-58.

Yang, Y.-Y., Chiou, J.-F., Hwang, G.-Y., Huang, I.-J., and Li, J.K.-K. (1992a). Evolutionary analyses of five US bluetongue viruses using the cognate S2 genes. Virus Res. **25**, 241-249.

Yang, Y.-Y., Johnson, T.M., Mecham, J.O., Tam, J.P., and Li, J.K.-K. (1992b). Epitopic mapping of linear and conformation-dependent antigenic determinants on GP5 of five US bluetongue viruses. Virology **188**, 530-536.

Yang, Y.-Y., and Li, J. K.-K. (1992). Complete genomic sequence of the GP5 protein gene of bluetongue virus serotype 11 and 17. Virus Res. **23**, 164-171.

Yang, Y.-Y., and Li, J.K.-K. (1993). Glycosylation of the major outer capsid protein of bluetongue viruses. Virology **194**, 350-354.

Yeager, M., Dryden, K.A., Olsen, N.H., Greenberg, H.B., and Bakers, T.S. (1990). Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. J. Struct. Biol. **110**, 2133-2144.

Yu, Y., Fukusho, A., and Roy, P. (1987) Nucleotide sequence of the VP4 core protein gene (M4 RNA) of US bluetongue virus serotype 10. Nucleic Acids Res. **15**, 7206.

Zhao, Y., Thomas, C., Bremer, C., and Roy, P. (1994). Deletion and mutation analyses of bluetongue virus NS2 protein indicate that the amino but not the carboxy terminus of the protein is critical for RNA-protein interactions. J. Virol. **68**, 2179-2185.

CHAPTER II

CONSERVATION OF THE M1 (SEGMENT 4) GENE SEQUENCE AND OF A LEUCINE ZIPPER MOTIF IN VP4 AMONG FIVE US BLUETONGUE VIRUSES[1]

ABSTRACT

Full-length cDNA copies of the M1 (segment 4) genes of US bluetongue viruses serotype-2, -10, -11, -13, and -17 were selectively amplified using genomic double-stranded RNA segments from purified BTV virions as templates and a modified polymerase chain reaction (Clamp-R). They were cloned into pUC19 plasmids and both strands of several clones were sequenced. The length of all five M1 genes is 1981 nucleotides. The result is contrary to the M1 gene of serotype-10 sequenced by Yu et al. (1987), which has 2011 nucleotides. The 5'- and 3'-noncoding regions of M1 genes are identical among all serotypes. The plus sense strand of the BTV M1 gene, which encodes the VP4 protein, possesses a single, long open reading frame (ORF) with an initiation codon (ATG) at nucleotides #9-11 and a stop codon (TAA) at nucleotides #1941-1943. This ORF encodes for a protein of 644 amino acid residues with a predicted molecular weight of about 75 kDa and a pI of +7 to +7.9. A potential leucine

[1]BTV-2 and BTV-11 M1 genes were cloned and sequenced by Dr. E. Hayama and Ms. Y. Jeong, respectively.

zipper motif was detected near the carboxyl terminus of the deduced VP4 amino acid sequence. The phylogeny established using the sequences of these five cognate M1 genes is consistent with the results of our previous phylogenetic studies of cognate genome segments 5, 6, 8, 9, and 10. Serotype-10, -11, -13, and -17 are closely related and serotype-2 is the most distantly related among the five US BTV serotypes.

INTRODUCTION

Bluetongue virus (BTV), classified in the *Orbivirus* genus of the family *Reoviridae* (Borden *et al.*, 1971; Murphy *et al.*, 1971), is an arthropod-borne virus that causes the bluetongue disease in domestic and wild ruminants. This disease was first discovered in South Africa more than a century ago and then found in various other parts of the world after 1949. BTV is an icosahedral-shaped particle consisting of a double-layered protein coat. Its genome is composed of 10 segmented double-stranded RNAs (dsRNAs) (Verwoerd *et al.*, 1972). Each segment of the viral genome except the S4 gene encodes a single protein (Grubman *et al.*, 1983; Sanger and Mertens *et al.*, 1983; Roy, 1989). The double-layer viral protein coat contains two outer capsid proteins (VP2 and VP5) and five inner capsid

proteins (VP1, VP3, VP4, VP6, and VP7). Four nonstructural proteins (NS1, NS2, NS3, and NS3A) have also been detected in BTV-infected cells (Hyatt *et al.*, 1991) and by *in vitro* translation (Grubman *et al.*, 1983; Mertens *et al.*, 1984; Van Dijk and Huismans, 1988).

VP4, a minor component of the inner capsid of BTV, is encoded by the M1 (segment 4) gene. Little is known about the structure and function of VP4. To gain a better understanding and to investigate the potential biological function(s) of this protein as well as the evolutionary relationship of the five US bluetongue viruses, the M1 genes of BTV serotype-2, -10, -11, -13, and -17 were cloned and sequenced.

MATERIALS AND METHODS

Virus preparation

The seed stocks of the five US BTV serotypes were provided by Arthropod-Borne Animal Disease Research Laboratory of USDA. They were three times plaque-purified in L-929 as described by Kowalik and Li (1987). The viruses were propagated in baby hamster kidney (BHK-21) cells grown in roller bottles (Corning) at a multiplicity of infection (M.O.I.) of 1. Viral particles were purified by using sucrose gradients as described by Kowalik and Li

(1989) and Kowalik *et al.* (1990). BTV-infected cells were scraped from roller bottles 2 days postinfection and pelleted at 3000x g for 25 min. The pellets were then resuspended in NTE (200 mM Tris-HCl, pH 8, 100 mM NaCl, 2 mM EDTA) buffer containing 1% (w/v) Triton X-100 and 0.5% (w/v) deoxycholate. After 10 min of incubation at room temperature, insoluble particles were removed by centrifuging at 2000x g for 5 min at room temperature. The virus containing supernatant was then loaded onto a 40% (w/v)/60% (w/v) sucrose step gradient containing 200 mM Tris-HCl (pH 8) and centrifuged for 90 min at 37,500 rpm in a Beckman SW41Ti rotor. At the end of centrifugation, the partially purified viruses located at the interface of the two sucrose solutions were collected and resuspended in 200 mM Tris-HCl (pH 8) containing 1% (w/v) N-lauroyl sarcosine and 10 mM dithiothreitol (DTT). The virus suspension was incubated at 37° C for 30 min and pelleted through a 1-cm 40% (w/v) sucrose cushion as described above. The purified viruses were then resuspended in 200 mM Tris-HCl (pH 8) and kept at -20° C. Each individual serotype was confirmed by neutralization assays and by comparing the characteristics electrophorograms of the viral proteins and dsRNAs on 10% SDS-PAGE (Li *et al.*, 1989).

Preparation of genomic dsRNA

The total dsRNA was extracted from the viral particles by the SDS-KCl method according to Li *et al.* (1989) and Kowalik *et al.* (1990). SDS was added to the purified virus solution to a final concentration of 1% SDS. The mixture was then boiled for 1 min and chilled on ice for 5 min. An equal volume of 0.5 M KCl was added to the mixture, and the mixture was kept on ice for another 5 min. The viral proteins and genomic dsRNA were separated by centrifuging at 10,000x g for 10 min (4^0 C) in a microcentrifuge. The dsRNA in the supernatant was then purified by phenol/chloroform extraction and ethanol precipitation at -20^0 C for 1 hr. The dsRNA was pelleted by centrifuging at 10,000x g for 10 min. The pellet was washed with 70% ethanol and dried under vacuum. The dsRNA pellet was then dissolved in double distilled water (ddH_2O) and kept at -20^0 C until use.

Amplification of the cognate M1 cDNAs

The full-length cDNAs of M1 genes were amplified using the purified BTV dsRNA as templates by the Clamp-R method (Kowalik *et al.*, 1990). A pair of primers, containing sequences complementary to the termini of BTV-11 M1 gene (Kiuchi *et al.*, 1983) and anchored with a PstI restriction

enzyme site (5'-AGTCGACCTGCAGGTTAAAACATGCCTGAG-3' and 5'-AGTCGACCTGCAGGTAAGTTGTACATGCCC-3'), was used to specifically amplify the full-length cDNA of the M1 genes from each BTV-2, -10, -11, -13, and -17. A solution (20 µl total) containing 0.3 µg of dsRNA templates and 0.5 µg of primers was boiled for 4 min and then chilled on ice immediately. The template-primer mixture was then mixed with 1 µl of 10 mM dNTP, 5 µl of 10x buffer (100 mM Tris-HCl, pH 8.3, 0.5 M KCl, 15 mM $MgCl_2$, and 0.1% (w/v) gelatin), 0.5 µl (12.5 units) of avian myeloblastosis virus-reverse transcriptase (AMV-RT) (Promega), and 0.5 µl (2.5 units) of native Taq DNA polymerase. The mixture was then overlaid with 100 µl of mineral oil to prevent evaporation. The amplification was carried out in a programmable thermal cycle reactor (Ericomp Corp.). First-strand cDNA was synthesized at 42^0 C by AMV-RT for 1 hr and subsequently amplified by 35 cycles of amplification, which included denaturation (95^0 C for 2 min), annealing (37^0 C for 2 min), and extension (72^0 C for 10 min). A final extension was done at 72^0 C for 20 min.

Cloning of M1 cDNAs

The amplified full-length cDNAs of M1 genes were restricted with PstI endonuclease and electrophoresed through a 1% low melting point agarose gel with 0.5x TBE

buffer (1x TBE is 90 mM Tris-borate, pH 8, 2 mM EDTA). The band containing the full-length M1 cDNA was excised from the gel and melted at 55^0 C for 5 min. Fifteen µl of the gel solution containing M1 cDNA was ligated with PstI-digested pUC19 vector in a final 50 µl of ligation mixture [containing 5 units of T4 DNA ligase, 1x ligation buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 2.5 µg/ml BSA)] (NEB) at room temperature for 2 hr. Ten µl of the ligation mixture was mixed with 100 µl of 50 mM $CaCl_2$ and 30 mM $MgCl_2$ and then used to transform 100 µl of competent *E. coil* strain XL-1 blue. The mixture was incubated on ice for 40 min, heat shocked at 42^0 C for 2 min, and grown in 0.8 ml of LB (10 g tryptone, 5 g yeast extract, 5 g NaCl per litter) at 37^0 C for 1 hr. The *E. coli* culture was then plated on LB plates containing 1% agar, 50 µg/ml ampicillin, 0.1 mM isopropyl-1-thio-D-galactoside (IPTG), and 20 µg/ml 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal). After 16 hr incubation at 37^0 C, white colonies were picked and grown in LB containing ampicillin for plasmid isolation. About 1.5 ml of overnight *E. coli* culture was pelleted by centrifuged at 10,000x g for 20 sec at room temperature. The cells were resuspended in 100 µl of lysis buffer (25 mM Tris-HCl, pH 8, 10 mM EDTA, 50 mM glucose) and incubated at room

temperature for 5 min. The suspension was then mixed with 200 µl of freshly prepared alkaline solution (0.2 N NaOH, 1% SDS) and incubated on ice for 5 min. The mixture was then mixed with 150 µl of ice-cold potassium acetate (pH 4.8) and incubated on ice for 5 min. The plasmids were separated from the cellular debris by centrifuging at 10,000x g for 5 min at 4^0 C. RNAse A was added to the supernatant to a final concentration of 20 µg/ml to digest cellular RNA for 30 min at 37^0 C. An equal volume of phenol/chloroform was added to the mixture, vortexed, and centrifuged at 10,000x g for 2 min. The plasmids in the supernatant was mixed with 2.5x volume of cold 100% ethanol (-20^0 C) and incubated at -80^0 C for 1 hr. The plasmids were pelleted by centrifuging at 10,000x g for 10 min at 4^0 C and washed with 70% ethanol. The DNA pellet was dried under vacuum and dissolved in TE. The presence of the M1 gene insert in the plasmids was checked by digesting with PstI restriction enzyme and analyzed by 1% agarose gel electrophoresis.

DNA sequencing

Plasmids were purified from 50 ml of overnight *E. coli* culture as described above except that they were further purified with polyethylene glycol (PEG). Plasmids were then dissolved in 168 µl of ddH_2O and mixed with 32 µl of 5

M NaCl and then 200 µl of 13% PEG. The mixture was kept on ice for a minimum of 30 min and centrifuged at 10,000x g for 10 min at 4^0 C. The DNA pellet was then washed with 70% ethanol, dried under vacuum, then dissolved in TE and kept at -20^0 C until use.

One µg of plasmid DNA in 40 µl of water was mixed with 4 µl of denaturation solution (2 N NaOH, 2 mM EDTA) and kept at room temperature for 5 min. After the incubation, 16 µl of ddH_2O and 6 µl of 3 M sodium acetate (pH 4.6) were added to the denatured DNA solution sequentially. To precipitate the DNA, 150 µl of cold 100% ethanol (-20^0 C) was added to the mixture, mixed, and kept at -80^0 C for 20 min. The denatured DNA was pelleted by centrifuging at 10,000x g for 5 min. The pellet was rinsed with 70% ethanol (-20^0 C) and vacuum dried. DNA was then dissolved in 7 µl of ddH_2O and kept on ice until use. To start the annealing reaction, 1 µl of primer (0.5 pmole/µl) and 5 µl of reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$) were added to the 7 µl DNA solution. The primer-template mixture was kept at 37^0 C for 30 min. For the labeling reaction, 1 µl of 0.1 M DTT, 2 µl of diluted labeling mix (1.25 µM dGTP, 1.25 µM dCTP, and 1.25 µM dTTP) (USB), 0.5 µl of [a-^{35}S]-dATP (10 µCi/µl) (Amersham), and 2 µl of diluted sequenase (USB) were added to the primer-template

mixture; the reaction was carried out at room temperature for 5 min. The termination procedure was started by transferring 3.5 µl of the previous solution to each of the four termination mixes (ddATP, ddCTP, ddGTP, ddTTP), which were pre-warmed at 37^0 C and incubated at 37^0 C for 5 min. The reaction was stopped by adding 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol-FF). The DNA mixtures were denatured at 80^0 C for 2 min and chilled on ice immediately before loading into a 5% polyacrylamide sequencing gel (acrylamide : bis-acrylamide, 30:1) containing 7 M urea. Five µl from each of the mixtures were loaded into a single lane. Electrophoresis proceeded at 2000 volts with 1x TBE buffer until the desired separation was reached. The temperature of the sequencing gel was kept at 55^0 C during the entire run. The sequencing gel was soaked in fixing solution (5% acetic acid, 15% methanol) for 15 min to fix the DNA and remove urea. The gel was then dried using a slab gel drier (Bio-Rad, Model 483) for 2 hr at 80^0 C. The dried gel was exposed with Kodak XAR-5 film at room temperature for autoradiography. Both strands of the inserted genes of different clones from different preparations were sequenced.

Phylogenetic analysis

Molecular phylogeny was analyzed by the PILEUP program in the GCG software (Devereux *et al.*, 1984) using both the nucleotide and deduced amino acid sequences of the entire M1 genes.

RESULTS

The full-length cDNAs of 5 BTV M1 genes were amplified (Fig. 1) using the purified BTV dsRNA as templates by the Clamp-R method. The amplified cDNA has a similar size as that of M1 gene dsRNA segment (about 2000 bp). The cDNA was digested with PstI restriction enzyme and cloned into pUC19 plasmid for sequencing.

All of the cloned cDNA of M1 genes were full-length except BTV-2, which has one PstI restriction enzyme site at position #1786-1790. The M1 gene of BTV-2 was cloned as two fragments in pUC19 at PstI site.

The M1 gene of each US BTV is 1981 nucleotides. They are highly conserved and can be aligned without any gaps (Fig. 2). A single long, ORF was found in the M1 gene of BTV-2, -10, -11, -13, and -17 with a start codon (ATG) at nucleotides #9-11 and a stop codon (TAA) at nucleotides #1941-1943. This ORF has a coding capacity of 644 amino acids and is flanked with an 8-nucleotide noncoding region

at its 5'-end and a 38-nucleotide noncoding region at its 3'-end, respectively. Both ends of the noncoding regions perfectly matched among all five US BTV serotypes. The coding sequences of M1 genes of BTV-2, -10, -11, -13, and -17 could also be aligned without any gaps. Yu *et al.* (1987) reported that the sequence of BTV-10 M1 gene contained 2011 nucleotides instead of the 1981 nucleotides determined in this study. The difference is the presence of a direct repeat of 30 nucleotides at #1610-1639. This repeat was not found in the BTV-10 M1 gene examined in this study.

The deduced amino acid sequences of VP4 are aligned and shown in Fig. 3. There is a high degree of sequence identity (96-99%). Hydropathy analysis indicates conserved hydrophobic amino and hydrophilic carboxyl termini. The VP4 proteins have an estimated molecular weight of 75-76 kDa and a pI between 7 to 7.9 (Table 1). The predicted VP4 of BTV-2, -10, -11, -13, and -17 has 644 amino acid residues, 10 fewer than that of BTV-10 reported by Yu *et al.* (1987). The 10 extra amino acids of BTV-10 VP4 are resulted from the 30 extra nucleotides in the sequence. The deduced protein contains about 32% charged amino acid residues and very little cysteine and glutamine (Table 1).

Phylogenetic analysis of the five US BTV serotypes was performed using both nucleotide and deduced amino acid sequences as shown in Fig. 4. The results are summarized in Table 2. The nucleotide sequences of the M1 gene of the five US BTVs are highly conserved. The M1 genes of BTV-11 and -17 has the highest identity of 99.44% (only 10 out of 1981 nucleotides mismatches). The M1 gene of BTV-2 is the most distantly related among the five US BTVs (Table 2 and Fig. 4).

DISCUSSION

The full-length cDNAs of the M1 genes were amplified by PCR from all five US BTV serotypes-2, -10, -11, -13, and -17 (Fig. 1). The M1 genes of all five US BTVs are highly conserved containing 1981 nucleotides and could be aligned without any gaps (Fig. 2).

The M1 genes of BTV-11 and -17 had the highest identity of 99.44% (10 mismatches out of 1981 nucleotides). The M1 gene of BTV-2 is more distantly related to the other four US BTV serotypes than the four are among themselves (Table 2 and Fig. 4).

Even though the nucleotide identity among these five US BTV serotypes ranged between 89-99%, the amino acid sequence mismatches were less than 4%. The reason for this

difference is that most of the nucleotide mismatches are found in the third base codon position (Table 3). The M1 gene of BTV-2 has 68-71% relatedness to the other four serotypes in the third base codon position calculated by the method described by Wiener and Joklik (1987). The M1 genes among the BTV-10, -11, -13, and -17 are very closely related (>97% identity) and they have more than 93% identity even at the third base codon position of codons (Table 4). An interpertation of these analyses is that BTV-2 diverged from the ancestor of the other four US BTV. This result is consistent with the conclusion derived from our previous studies of segments 1, 3 5, 6, 8, 9, and 10 (Hwang *et al.*, 1992a; 1992b; Yang *et al.*, 1992; Yang and Li, 1992; Hwang *et al.*, 1993; 1994; Huang *et al.*, 1995).

In this study, we reported the M1 genes contain 1981 nucleotides instead of 2011 reported in BTV-10 by Yu *et al.* (1987). We concluded that the difference is due to a direct repeat of 30 nucleotides at #1610-1639. The repeat also caused the increase of 10 amino acid residues of BTV-10 VP4 that they had reported. This was not found in the BTV-10 sequence of this study.

Analysis of the deduced amino acid sequences of VP4 revealed several interesting predicted structures and functions. Potential amidation and glycosylation sites

were found at position #106-109 (IGRR) and #459-462 (NGSY) of all five serotypes, respectively. Six potential N-myristylation sites were also found in VP4 at position #297-302 (GGAFSS), #298-303 (GAFSSA), #419-424 (GLEPSI), #548-553 (GLDLSG), #562-567 (GAYVTD), and #627-632 (GMREGA). Potential cAMP- and cGMP-dependent protein kinase phosphorylation sites could also be located at position #208-211 (KKRS) of all five BTV serotypes. Many more potential phosphorylation sites for casein kinase II phosphorylation, protein kinase C phosphorylation, and tyrosine kinase phosphorylation are found in VP4. However, NS2 is the only reported phosphorylated BTV viral protein (Devaney *et al.*, 1988; Huismans *et al.*, 1987).

Two regions at residues #296-299 (DRLG) and #550-553 (DLSG) of all five US BTV VP4 proteins have the DX_2G sequence motif, which is conserved in all GTPase (Bourne *et al.*, 1991). In the study of $p21^{ras}$, products of the *ras* oncogenes and proto-oncogenes, the asparate binds the catalytic Mg^{2+} through an intervening water molecule, whereas the amide proton of the glycine forms a hydrogen bond with the r-phosphate of GTP. These regions may be involved in the GTP-binding activity of VP4.

A potential leucine-zipper motif located at positions #523-555 (**L**RVESSV**L**RVRNPT**L**HETADE**L**RVRNPT**L**HETADE**L**QRMGLD**L**)

contains a repeat of leucine residues at every seventh position (Fig. 5) and a short stretch of adjacent charged amino acid residues. This is the first leucine-zipper motif reported in dsRNA viruses. This motif is predicted to form an amphipathic α-helix. An unusual cluster of proline residues (5 out of 9) was also located about 10 amino acids before this unique motif. A similar proline-rich region was identified as a Src homology 3 (SH3) binding site of the SH3 binding protein (Ren *et al.*, 1993). The proline-rich region of VP4 may serve a similar function as a protein-protein association module to facilitate binding to other viral proteins.

The potential leucine-zipper motif of VP4 resembles that of many known transcriptional transactivators (Landschulz *et al.*, 1988), which have a heptad repeat of five leucine residues and a basic region adjacent to the leucine repeats. Like *jun,* this region has uncharged residues at the fifth position of the repeat. It also has uncharged residues at every first and fifth positions, which have been reported to stabilize the zipper in a coiled-coil helical conformation (O'Shea *et al.*, 1989; Hu *et al.*, 1990). The first and the fifth positions of this heptad are usually occupied by hydrophobic amino acids (the 4-3 rule) (Zhou *et al.*, 1992). Thus one side of the helix

is predominantly hydrophobic. Another model of a coiled-coil has symmetrical secondary hydrogen-bonding between the polar side chain of one helix and the peptide backbone of the opposite chain (Tropsha *et al.*, 1991). Although helix-breaking proline and glycine residues are found in this region of the VP4 leucine-zipper motif, a single glycine inserted by mutation did not abolish the function of yeast GCN4 protein, which is a DNA-binding protein with a leucine-zipper motif (Pu and Struhl, 1991). The *T. thermophila* isoleucyl-tRNA synthetase (ileRSs) has two leucine-zipper motifs in the carboxyl one third of the protein (Csank and Martindale, 1992) that are conserved in the other ileRSs. Proline residues are also found in these motifs. In a recent report by Xia and Lai (1992), the hepatitis delta antigen (HDAg) with a leucine-zipper-like motif containing a proline residue is involved in the replication of HDV RNA. The results of this study demonstrated that the leucine-zipper motif is responsible for HDAg dimerization and the motif on the HDAg is indispensable for its activity in HDV RNA replication.

Although the potential leucine-zipper motif found in the BTV VP4 does not match all of the criteria of a DNA-binding protein with a leucine zipper motif (bZIP), VP4 shares several features with proteins involved in RNA-

protein interaction (Pu and Struhl, 1991; Xia and Lai, 1992). These RNA-binding proteins have the potential to form an amphipathic α-helix, in which a proline residue(s) is found inside the heptad, and a basic region is located downstream of the motif.

Recently, VP4 expressed in insect cells in a baculovirus expression system has been suggested as the guanylyltransferase involved with capping BTV mRNAs (Le Blois *et al.*, 1992). However, this has not been definitely proven. The characteristics of VP4 suggest that VP4 might play an important role in BTV genome transcription and replication. The biological function(s) of VP4 is still unknown but the investigation of its potential function(s) is currently in progress.

REFERENCES

Borden, E.C., Shope, R.E., and Murphy, F.A. (1971). Physiochemical and morphological relationship of some arthropod-borne viruses to bluetongue virus--a new taxonomic group: Physiochemical and serological studies. J. Gen. Virol. **13**, 261-271.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1991). The GTPase superfamily: Conserved structure and molecular mechanism. Nature **349**, 117-127.

Csank, C., and Martindale D.W. (1992). Isoleucyl-tRNA synthetase from the ciliated protozoan *tetrhymena thermophila*. J. Biol. Chem. **260**, 4592-4599.

Devaney, M.A., Kendall, J., and Grubman, M.J. (1988). Characterization of a nonstructural phosphoprotein of two orbiviruses. Virus Res. **11**, 151-164.

Devereux, I., Haeberli, J., and Smithies, H. (1984). A comprehensive set of sequence analysis program for the VAX. Nucleic Acid Res. **12**, 387-395.

Grubman, M.J., Appleton, J.A., and Letchworth, G.J. (1983). Identification of bluetongue virus serotype 17 genome segments coding for polypeptides associated with virus neutralization and intergroup reactivity. Virology **131**, 353-366.

Hu, J.C., O'Shea, E.K., Kim, P.S., and Sauer, R.T. (1990). Sequence requirements for coiled-coils: Analysis with lambda repressor-GCN4 leucine zipper fusions. Science **250**, 1400-1403.

Huang, I-J., Hwang, G.-Y., Yang, Y.-Y., Hayama, E., and Li, J.K.-K. (1995). Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five US bluetongue viruses. Virology **214**, 280-288.

Huismans, H., Van Dijk, A.A., and Bauskin, A.R. (1987). *In vitro* phosphorylation and purification of a nonstructural protein of bluetongue virus with affinity for single-stranded RNA. J. Virol. **61**, 3589-3595.

Hwang, G.-Y., Chiou, J.-F., Yang, Y.-Y., and Li, J. K.-K. (1992a). Sequence conservation among the cognate nonstructural NS3/3A protein genes of six bluetongue viruses. Virus Res. **23**, 151-161.

Hwang, G.-Y., Chiou, J.-F., Yang, Y.-Y., and Li, J. K.-K. (1992b). Comparative sequence analysis of the cognate structural protein VP6 genes of five US bluetongue viruses. Virus Res. **24**, 315-323.

Hwang, G.-Y., Chiou, J.-F., Yang, Y.-Y., and Li, J. K.-K. (1993). High sequence conservation among the United States bluetongue viruses cognate M2 genes which encode the nonstructural NS1 tubule protein. Virology **192**, 321-327.

Hwang. G.-Y., Xiang, M., and Li, J.K.-K. (1994). Analyses and conservation of sequences among the cognate L3

segments of the five United States bluetongue viruses. Virus Res. **32**, 381-389.

Hyatt, A.D., Gould, A.R., Coupar, B., and Eaton, B.T. (1991). Localization of the non-structural protein NS3 in bluetongue virus-infected cells. J. Gen. Viol. **72**, 2263-2267.

Kiuchi, A., Rao, C.D., and Roy, P. (1983). Analyses of bluetongue viral RNA sequences. In: Double-stranded RNA viruses (R.W. Compans and D.H.L. Bishop, eds.), pp. 55-64. Elsevier, New York.

Kowalik, T.F., and Li, J.K.-K. (1987). The genetic relatedness of United States prototype bluetongue viruses by RNA/RNA hybridization. Virology **158**, 276-284.

Kowalik, T.F., and Li, J.K.-K. (1989). Sequence analysis and structural predictions of double-stranded RNA segments S1 and VP7 from United States prototype bluetongue virus serotype 13 and 10. Virology **172**, 189-195.

Kowalik, T.F., Yang, Y.-Y., and Li, J.K.-K. (1990). Molecular cloning and comparative sequence analyses of bluetongue virus S1 segments by selective synthesis of specific full-length DNA copies of dsRNA genes. Virology **177**, 820-823.

Landschulz, W.H., Johnson, P.F., and McKnight, M.C. (1988). The leucine-zipper: A hypothetical structure common to a new class of DNA-binding proteins. Science **240**, 1759-1764.

Le Blois, H., French, T. Mertens, P.P.C., Burroughs, J.N., and Roy, P. (1992). The expressed VP4 protein of bluetongue virus binds GTP and is the candidate guanylyl transferase of virus. Virology **189**, 757-761.

Li, J.K.-K., Johnson, T., Yang, Y.-Y., and Shore, V. (1989). Selective separation of virus proteins and dsRNA by SDS-KCl precipitation. J. Virol. Methods **26**, 3-16.

Mertens, P.P.C., Brown, F., and Sanger, D.V. (1984). Assignment of the genome segments of bluetongue virus type 1 to the proteins which they code. Virology **135**, 207-217.

Murphy, F.A., Borden, E.C., Shope, R.E., and Harrison, A. (1971). Physiochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus--a new taxonomic group: Electron microscopic studies. J. Gen. Virol. **13**, 273-278.

O'Shea, E.K., Rutkowski, R., and Kim, P.S. (1989). Evidence that the leucine zipper is a coiled-coil. Science **243**, 538-542.

Pu, W.T., and Struhl, K. (1991). The leucine zipper symmetrically positions the adjacent basic regions for specific DNA-binding. Proc. Natl. Acad. Sci. USA **88**, 6901-6905.

Ren, R., Mayer, B.J., Cicchetti, P., and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science **259**, 1157-1161.

Roy, P. (1989). Bluetongue virus genetic and genome structure. Virus Res. **13**, 179-206.

Sanger, D.V., and Mertens, M.J. (1983). Comparison of type 1 bluetongue virus protein synthesis *in vivo and in vitro*. In: Double-stranded RNA Viruses (R.W. Compans and D.H.L. Bishop, eds.), pp. 183-191. Elsevier, New York.

Tropsha, A., Bowen, J.P., Brown, F.K., and Kizer, J.S. (1991). Do interhelical side chain-backbone hydrogen bonds participate in formation of leucine zipper coil coils? Proc. Natl. Acad. Sci. USA. **88**, 9488-9492.

Van Dijk, A.A., and Huismans, H. (1988). *In vitro* transcription and translation of bluetongue virus mRNA. J. Gen. Virol. **69**, 573-581.

Verwoerd, D.W., Els, H.J., DeVilliers, E.M., and Huismans, H. (1972). Structure of the bluetongue virus capsid. J. Virol. **10**, 783-794.

Wiener, J.R., and Joklik, W.K. (1987). Comparison of the reovirus serotype 1, 2, and 3 sigma 3 genome segments encoding the nonstructural protein sigma NS. Virology **161**, 332-339.

Xia, Y.-P., and Lai, M.M.C. (1992). Oligomerization of hepatitis delta antigen is required for both the trans-

activating and trans-dominant inhibitory activities of the delta antigen. J. Virol. **66**, 6641-6648.

Yang, Y.-Y, Hwang, G.-Y., Chiou, J.-F., Huang, I-J., and Li, J. K.-K. (1992). Evolutionary analysis of five US bluetongue viruses using the cognate S2 genes. Virus Res. **25**, 241-249.

Yang, Y.-Y., and Li, J. K.-K. (1992). Complete genomic sequence of the GP5 protein gene of bluetongue virus serotypes 11 and 17. Virus Res. **23**, 163-171.

Yu, Y., Fukusho, A., and Roy, P. (1987). Nucleotide sequence of the VP4 core protein gene (M4 RNA) of US bluetongue virus serotype 10. Nucleic Acids Res. **15**, 7206.

Zhou, N.E., Kay, C.M., and Hodges, R.S. (1992). Structural model proteins: The relative contribution of leucine residues at the nonequivalent positions of the 3-4 hydrophobic repeat to the stability of the two-stranded α-helical coiled-coil. Biochemistry **31**, 5739-5746.

TABLE 1

Amino Acid Content of Deduced VP4 of the Five

US Bluetongue Viruses

	BTV-2	BTV-10	BTV-11	BTV-13	BTV-17
Ala (A)	38	38	39	39	38
Cys (C)	4	5	5	5	5
Asp (D)	46	49	48	48	48
Glu (E)	45	45	46	46	45
Phe (F)	24	25	25	24	25
Gly (G)	34	34	33	33	33
His (H)	26	25	25	24	25
Ile (I)	40	37	37	38	39
Lys (K)	38	36	37	36	37
Leu (L)	62	62	62	62	60
Met (M)	27	27	28	26	27
Asn (N)	22	23	24	25	24
Pro (P)	29	30	30	30	31
Gln (Q)	9	8	8	9	8
Arg (R)	53	57	55	53	56
Ser (S)	41	36	36	37	38
Thr (T)	20	23	22	22	22
Val (V)	44	41	41	42	40
Trp (W)	15	15	15	15	15
Tyr (Y)	27	28	28	30	28
A+G	72	72	72	72	71
S+T	61	59	58	59	60
D+E	91	94	94	94	93
D+E+N+Q	122	125	126	128	125
H+K+R	117	118	117	113	118
D+E+H+K+R	208	212	211	207	211
I+L+M+V	173	167	168	168	166
F+W+Y	66	68	68	69	68
pI	7.82	7.58	7.43	7.06	7.76
MW (kDa)	75.1	75.4	75.4	75.3	75.4

TABLE 2

Sequence Identity Matrix of the M1 (Segment 4) Genes among the Five US Bluetongue Viruses

	Identity of sequences (%)				
	BTV-2	BTV-10	BTV-11	BTV-13	BTV-17
BTV-2		89.30	89.55	89.10	89.30
BTV-10	97.36		98.69	98.44	98.49
BTV-11	97.52	99.69		98.38	99.44
BTV-13	96.74	98.91	99.07		98.13
BTV-17	97.52	99.69	99.84	99.07	

Note. Numbers above the diagonal indicate the percentage of identity of nucleotide sequences and numbers below the diagonal indicate the percentage of identity of amino acid sequences.

TABLE 3

Number of Mismatches Between BTV M1 (Segment 4) Nucleotide Sequences Based on Codon Position

Codon positions	BTV-17 mismatches	%	BTV13 mismatches	%	BTV-11 mismatches	%	BTV-2 mismatches	%
BTV-10								
1st	4	<1	6	<1	4	<1	32	5
2nd	2	<1	4	<1	2	<1	19	3
3rd	26	4	22	4	21	4	161	25
BTV-17								
1st			4	<1	2	<1	32	5
2nd			2	<1	0	0	16	2
3rd			32	5	8	2	165	23
BTV-13								
1st					4	<1	34	5
2nd					2	<1	18	2
3rd					26	4	164	23
BTV-11								
1st							31	5
2nd							18	2
3rd							156	22

TABLE 4

Comparison of the Nucleotide Sequence Divergence Pattern of BTV M1 (Segment 4) Genes Based on Codon Positions

Serotype	Base codon position	Number of matched nucleotides	Number of true matches[a] /% of true matches[b]	Extent of relatedness[c]
2 vs. 10	1	613/644	452/70	94
	2	629	468/72	97
	3	494	333/51	69
2 vs. 17	1	613/644	452/70	94
	2	632	471/73	98
	3	495	334/52	69
2 vs. 13	1	611/644	450/70	93
	2	630	469/73	97
	3	494	333/52	69
2 vs. 11	1	614/644	453/70	94
	2	630	469/73	97
	3	504	343/53	71
Among BTV-10, -11, -13, and -17	1	-	-	>98
	2	-	-	>98
	3	-	-	>93

a. Number of matches minus the number of expected matches among random sequences which is 161 in M1 genes (25% of 644).

b. Ratio of the number of true matches to the total number of nucleotides (percentage). A value of 75% and 0% represents complete identity and divergence, respectively.

c. Ratio of number of true matches divided by (644-161) (percentage).

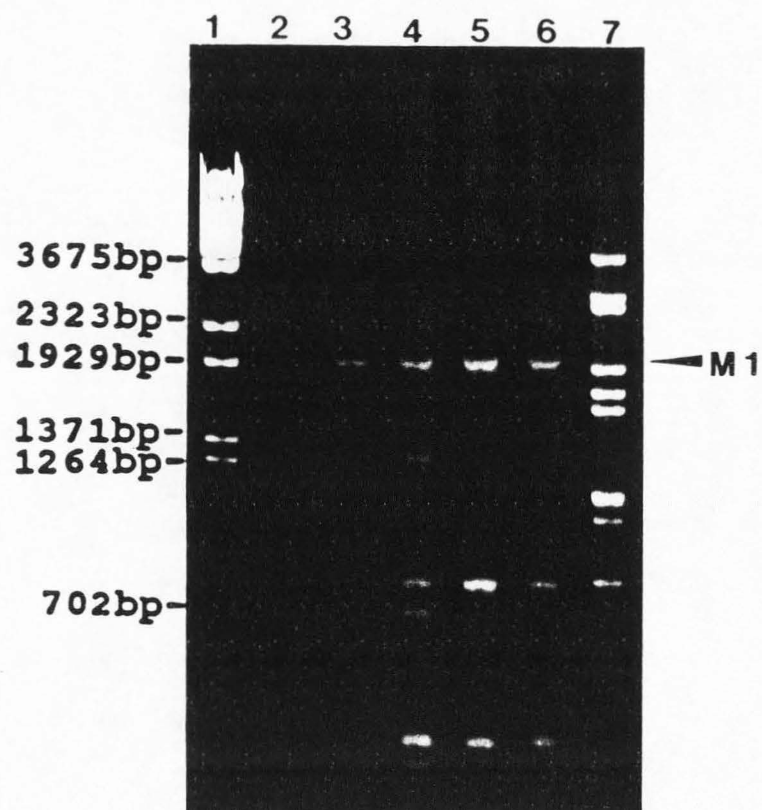


FIG. 1. Amplification and synthesis of full-length cDNA of M1 (segment 4) genes of five US BTV serotypes. Total genomic BTV dsRNA was mixed with primers (see text) complementary to the 3'-ends of M1 gene. The dsRNA was reverse transcribed into cDNA and then amplified by PCR. Ten percent of the final product was analyzed by 1% agarose gel electrophoresis. Lane 1: 0.5 µg of BstE II digested lambda DNA marker; Lane 2: BTV-2; Lane 3: BTV-10; Lane 4: BTV-11; Lane 5: BTV-13; Lane 6: BTV-17; Lane 7: 0.3 µg BTV-13 genomic dsRNA.

```
BTV10 GTTAAAACATGCCTGAGCCACACGCCAGTACTCTATGTCACAAATGAGCTCTCTCATATCGTTAAGGATGGATTTTTGCCA 80
BTV17 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A••••••••••••••
BTV13 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A••••••••••••••
BTV11 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A••••••••••••••
BTV 2 ••••••••••••••••••••••••••••••••••••••••••C•••••••••••C••••••••••AG•••••••••••••

BTV10 ATATGGAAACTAACTGGAGATGAGAGTTTAAATGACTTATGGCTTGAAAATGGAAAGTACGCGACAGACGTATATGCGTA 160
BTV17 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A••
BTV13 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A••
BTV11 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A••
BTV 2 •••••••G•T••••••••••TA•••••C••••••TAC•••••••••••••••••••A••T•••••T••T••••••••A••

BTV10 CGGAGACGTTTCAAAGTGGACGATACGACAATTACGGGGTCATGGATTCATTTTTATAAGCACGCACAAGAACGTACAGC 240
BTV17 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••
BTV 2 ••••••T••A••••••••••••••••G•••C•••••••••••••••••••••••C•••••T••A••••••••TA••••A•

BTV10 TAGCTGACATTATTAAGACGGTTGACGTCCGTATTCCTCGGGAGGTTGCAAGAAGTCATGATATGAAAGCTTTTGAAAAT 320
BTV17 •••••••••••••••••••••••••••••••••••••••••••••••••••G••C•••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••
BTV11 •••••••••••••••••••••••••••••••••••••••••••••••••••G••••••••••••••••••••••••••••
BTV 2 •G••••••••C•••••A•••••C•T•••••••••••••••••A••C••••A•••••••A•••••••••••A•••••••••

BTV10 GAGATCGGCAGACGCAGGATCCGTATGCGCAAGGGATTTGGTGATGCATTGAGAAATTACGCCTTTAAGATGGCTATCGA 400
BTV17 ••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 ••A••T••T••••••••A••••••••••T•••••••••••••••••GC••••G•••A•GCT•••••••••••••••••••

BTV10 ATTTCACGGGTCGGAGGCGGAAACGTTGAACGATGCAAATCCTCGGTTACATAAAATTTATGGAATGCCGGAAATACCGC 480
BTV17 •••••••••••••••••••••••••••••••••••••••••••••C••••••••••••••••••••••••••••••••••
BTV13 •••••••••A•••••••••••••••••••••••••••••••••••C•••••••••G••••••••••••••••••••••••
BTV11 •••••••••••••••••••••••••••••••••••••••••••••C•••••••••••••••••••••••••••••C••••
BTV 2 G••••••••••••••A•••••G••••A•••••••••••••••••A••G•••••••••••••••••••••••••••C••••

BTV10 CATTATACATGGAATATGCGGAAATAGGGACTAGATTTGACGATGAGCCGACTGATGAAAAGTTAGTATCAATGCTTGAT 560
BTV17 ••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••G•••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••
BTV 2 ••C•••••••T•••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••G•••••••••

BTV10 TATATCGTTTACAGTGCCCGAAGAGGTGCACTATGTTGGATGTGGTGACCTACGTACCCTAATGCAGTTCAAGAAACGATC 640
BTV17 ••••••••••••••C•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••C•••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 ••••••••••••••C••A••G••••A••••••••A•••••C••••••••••••••••A•T•••••••••••A•••••T••
```

FIG. 2. Nucleotide sequence alignment of the M1 (segment 4) genes of five US bluetongue viruses. The initiation (ATG) and termination codons (TAA) are indicated by boxes. A total of 1981 nucleotides were found in the M1 genes of BTV-2, -10, -11, -13, and -17. Asterisks represent nucleotides identical to those of BTV-10. Thymine (T) is shown in place of uracil presented in the genomic RNA.

```
BTV10 ACCAGGACGGTTTAGAAGGGTGTTATGGCACGTATATGACCCAATAGCACCTGAGTGTTCAGATCCAAACGTTATAGTTC 720
BTV17 ••••••••••••••••••A•••••••••••••••••••••••••••••G•••••••••••••••••G•••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••A•••••••••••••••••••••••••••••G•••••••••••••••••G•••••••••••••
BTV 2 •••••••••••••••••••••A•••••••••••••••C•T•••••••••••G••A•C•••••••••••••••••••••••

BTV10 ATAATATTATGGTGGATTCAAAGAAAGACATTTTGAAACATATGAATTTTTTGAAACGTGTTGAGAGACTTTTCATATGG 800
BTV17 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 •••••G••••••••••••••••••••••••••••••••••••••••C•••••A•••••••••••A••G••C•••••••••

BTV10 GATGTATCGTCAGATCGATCGCAGATGAATGATCATGAGTGGGAGACGACCAGGTTTGCGGAGGATAGATTGGGTGAGGA 880
BTV17 •••••••••••••••••••••••••••••••••••••••••••••••A••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••••A••A••••••••••••••••••••••••••••••••
BTV11 •••••••••••••••••••••••••••••••••••••••••••••••A••••••••••••••••••••••••••••••••
BTV 2 •••••G••A••••••••••••••••••G•••••G••••A•••••AT•••••••A••C••A••••••••G•••••••••••

BTV10 AATAGCTTATGAAATGGGTGGTGCATTTTCCAGTGCATTGATCAAGCACAGGATACCGAATTCAAAAGACGAATATCACT 960
BTV17 ••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••G•••••••
BTV13 •••••••••••••••••••••••••••••••••C••••••••T••A••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••••••••••••••G•••••••
BTV 2 G•••••••••••••••••C•••••G••C••••••••••••••A••A••T••••••••A••••••G•••••••G•••••••

BTV10 GCATTTCGACCTACTTATTCCCCCAACCGGGGGCCGATGCGGATATGTATGAATTAAGGAATTTCATGAGATTGAGAGGT 1040
BTV17 ••••••••••T•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••T•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 ••••••••••T•••C•TC•••••••G••••••••T••••••••••••••C•••••••••••••••••••••••••A••••

BTV10 TACTCACACGTGGATCGCCACATGCATCCAGACGCTTCCGTGACGAAAGTTGTTTCACGTGATGTGCGCAAAATGGTCGA 1120
BTV17 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••G•••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••••••••••C•••••T•••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••G•••••••••••••••••••••••
BTV 2 ••••••••T••A••••••••T•••••C•••••T••A•••••A•T••••••••••••G••••••••A••••••••••••••

BTV10 ATTGTATCACGGTCGCGATCGTGGTAGATTTCTAAAAAAGAGACTATTTGAACACCTTCATATTGTACGTAAGAATGGAT 1200
BTV17 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••G•••••A•••••••
BTV13 ••••••••••••••••••••••••••C•••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 •••••••••T••••••••••••••C••••••G•G••G••C••G••••••••••••••••••••C••••••••••••••••

BTV10 TGTTACACGAAAGTGATGAGCCACGAGCGTATCTGTTTTATTTGACCAATCGGTGCAATATGGGATTGGAGCCTAGTATT 1280
BTV17 •••••••••••••••••••••••••••••G••••••••••••••••••••••••••••C•••••••••••••••••••••
BTV13 •••••••••••••••••••••••••••••G••••••••••••••••••••••••••••C•••••••••••••••••••••
BTV11 •••••••••••••••••••••••••••••G••••••••••••••••••••••••••••C•••••••••••••••••••••
BTV 2 ••C•G••T•••••••••••A••••••••AG••••••••••••••••••••••••••••C••••••C••••A••A••C•••

BTV10 TATGAGGTAATGAAGAAGTCGGTGATAGCTACTGCTTGGGTGGGCCGTGCGCCTTTATATGATTATGATGATTTCGCGTT 1360
BTV17 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••C•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 •••••A••••••••••••••••••••C•••••A••C•••••A••••••••T•••C•••••••••••••••••••••••••

BTV10 ACCCAGATCTACCGTTATGCTCAACGGATCCTACCGCGATATCAGAATTCTAGATGGCAATGGCGCAATCCTATTCCTAA 1440
BTV17 •••••••••••••••••••••••••••••••••••••••C••••••••••••••••••••••••••••••••••••••••
BTV13 •••••••••••••••••••••••••••••••••••••••••••••T••••••••••••••••••••••••••••A•••••
BTV11 •••••••••••••••••••••••••••••••••••••••C••••••••••••••••••••••••••••••••••••••••
BTV 2 ••••••••••••T••C••••••••T•••••T••••AT••••••••••••••••••••T•••••••••••••••••T••••

BTV10 TGTGGAGGTACCCGGATATCGTAAAGAAAGATTTGACGTATGACCCTGCGTGGGCGATGAATTTTGCTGTTTCGCTAAAG 1520
BTV17 •••••••••••••A••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 •••••••••••••A•••••••••••••••••••••••••••••••••••A••••••••••••••••••••••••••••••
BTV11 •••••••••••••A••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 ••••••A••••••A••C••T••G•••••••••••A••A•••••••A•••••••••A••••••••C•••••C•••T•••••
```

FIG. 2-Continued

```
BTV10 GAGCCGATACCTGATCCTCCTGTGCCTGATATTTCTTTGTGTAGGTTCATCGGACTGCGCGTCGAATCATCCGTGCTGAG 1600
BTV17 ••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••G•••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••C•••••••••••••••••••••••••••••T•••••••••••••••••
BTV11 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••T•••••••••••••••••
BTV 2 •••••••••••C•••••C••••••••C•••••C••CC•A••C••A••T••••••••••••••T••G•••••T•••T••••

BTV10 GGTCCGAAACCCAACATTACATGAGACGGCTGACGAACTGAAACGGATGGGATTGGATTTGTCTGGTCATTTATATGTCA 1680
BTV17 •••••••••T•••••••••••C••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 •••••••••T••••••••••••••A•••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 ••••••••••••••••••••••••A••••••••T•••••A••••••••••••••••••••A•••••••••C••••C••••

BTV10 CATTAATGTCCGGCGCTTATGTCACAGATCTGTTTTGGTGGTTTAAGATGATTCTAGATTGGTCTGCGCAAAACAGGGAG 1760
BTV17 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••A
BTV13 ••••••••••••••••••••••••••••••••••••••••••••••C••C•••••••••••••••••••••••••A•••A
BTV11 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••A•••A
BTV 2 ••••G•••••••••••••••••••••••••••••C••••••••C•••••••••••C••••••••••••••••G••A••••

BTV10 CAAAAACTACGCGATCTAAAGAGGTCTGCGGCGGAAGTAATTGAGTGGAAGGAGCAGATGGCCGAGCGTCCATGGCATGT 1840
BTV17 •••••••••••••••••••••••••••••••••••G••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 •••••••••••••••••••••••••••••••••••G••••••••••••••••••••••••••••••••••••••••••••
BTV 2 ••••••T••••T•••T•••••••••••••A•••••G•••••C••A•••••••••••••••••••••••••••••••C•••

BTV10 GAGAAATGATCTGACTCGTGCGCTAAGGGAATACAAACGGAAAATGGGGATGAGAGAGGGAGCCTCGATTGATTCGTGGC 1920
BTV17 ••••••••••••••T•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 ••••••••••••••T•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 ••••••••••••••T•••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 •••••••AG•••A•T•GCA•••T•G•••••G••••••••••••••••••••A•••••••••••••••••C••••••••••

BTV10 TAGAATTACTGCGTCACTTATAATGCGTGACTGCTAGGTGAGGGGGGCATGTACAACTTAC   1981
BTV17 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV13 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV11 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
BTV 2 •••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
```

FIG. 2-Continued

```
BTV10 MPEPHAVLYVTNELSHIVKDGFLPIWKLTGDESLNDLWLENGKYATDVYAYGDVSKWTIRQLRGHGFIFISTHKNVQLAD 80
BTV17 *******************N************************************************************
BTV13 *******************NE***********************************************************
BTV11 ********************E***********************************************************
BTV 2 *******************S******R***V****V***************************************I****

BTV10 IIKTVDVRIPREVARSHDHKAFENEIGRRRIRMRKGFGDALRNYAFKMAIEFHGSEAETLNDANPRLHKIYGMPEIPPLY 160
BTV17 ********************************************************************************
BTV13 *********************************************************************V**********
BTV11 ***************************************************************************M****
BTV 2 **************K*Q**************************KL******************************M****

BTV10 MEYAEIGTRFDDEPTDEKLVSMLDYIVYSAEEVHYVGCGDLRTLMQFKKRSPGRFRRVLWHVYDPIAPECSDPNVIVHNI 240
BTV17 ********************************************************************************
BTV13 *******A************************************************************************
BTV11 ********************************************************************************
BTV 2 I*****************************************I********************H*****S*********V

BTV10 MVDSKKDILKEDGNFLKRVERLFIHDVSSDRSQHNDHEWETTRFAEDRLGEEIAYEMGGAFSSALIKHRIPNSKDEYHCIS 320
BTV17 ********************************************************************************
BTV13 **********Y*********************************************************************
BTV11 ******N*************************************************************************
BTV 2 ********************************D*D***S*********************************R*******

BTV10 TYLFPQPGADADHYELRNFMRLRGYSHVDRHKHPDASVTKVVSRDVRKMVELYHGRDRGRFLKKRLFEHLHIVRKNGLLH 400
BTV17 ********************************************************************************
BTV13 **********************************************************T*********************
BTV11 ********************************************************************************
BTV 2 ***L******************K**************M**********************V*M*****************

BTV10 ESDEPRADLFYLTNRCNMGLEPSIYEVMKKSVIATAWVGRAPLYDYDDFALPRSTVMLNGSYRDIRILDGNGAILFLMWR 480
BTV17 ********************************************************************************
BTV13 *****************************************************************S*********Y****
BTV11 ********************************************************************************
BTV 2 *************************************************************H*****************K

                                                v      v      v      v      v
BTV10 YPDIVKKDLTYDPAWAKNFAVSLKEPIPDPPVPDISLCRFIGLRVESSVLRVRNPTLHETADELKRMGLDLSGHLYVTLM 560
BTV17 ********************************************************************************
BTV13 ***************************************************************Q****************
BTV11 ********************************************************************************
BTV 2 ***********H********************************************************************

BTV10 SGAYVTDLFWNFKMILDWSAQNREQKLRDLKRSAAEVIEWKEQHAERPWHVRNDLTRALREYKRKMGMREGASIDSWLEL 640
BTV17 **********************K********************************I************************
BTV13 ************NI********K********K***********************I************************
BTV11 **********************K********************************IA***********************
BTV 2 *********************SK******************************S*IA**********I************

BTV10 LRHL 644
BTV17 ****
BTV13 ****
BTV11 ****
BTV 2 ****
```

FIG. 3. Alignment of the deduced amino acid sequences of the VP4 proteins of the five US bluetongue viruses. The asterisks represent amino acids identical to those of BTV-10. A total of 644 amino acids were found in the VP4 of BTV-2, -10, -11, -13, and -17. The seventh leucines of the potential leucine-zipper motif (#523-551) are indicated by arrows.

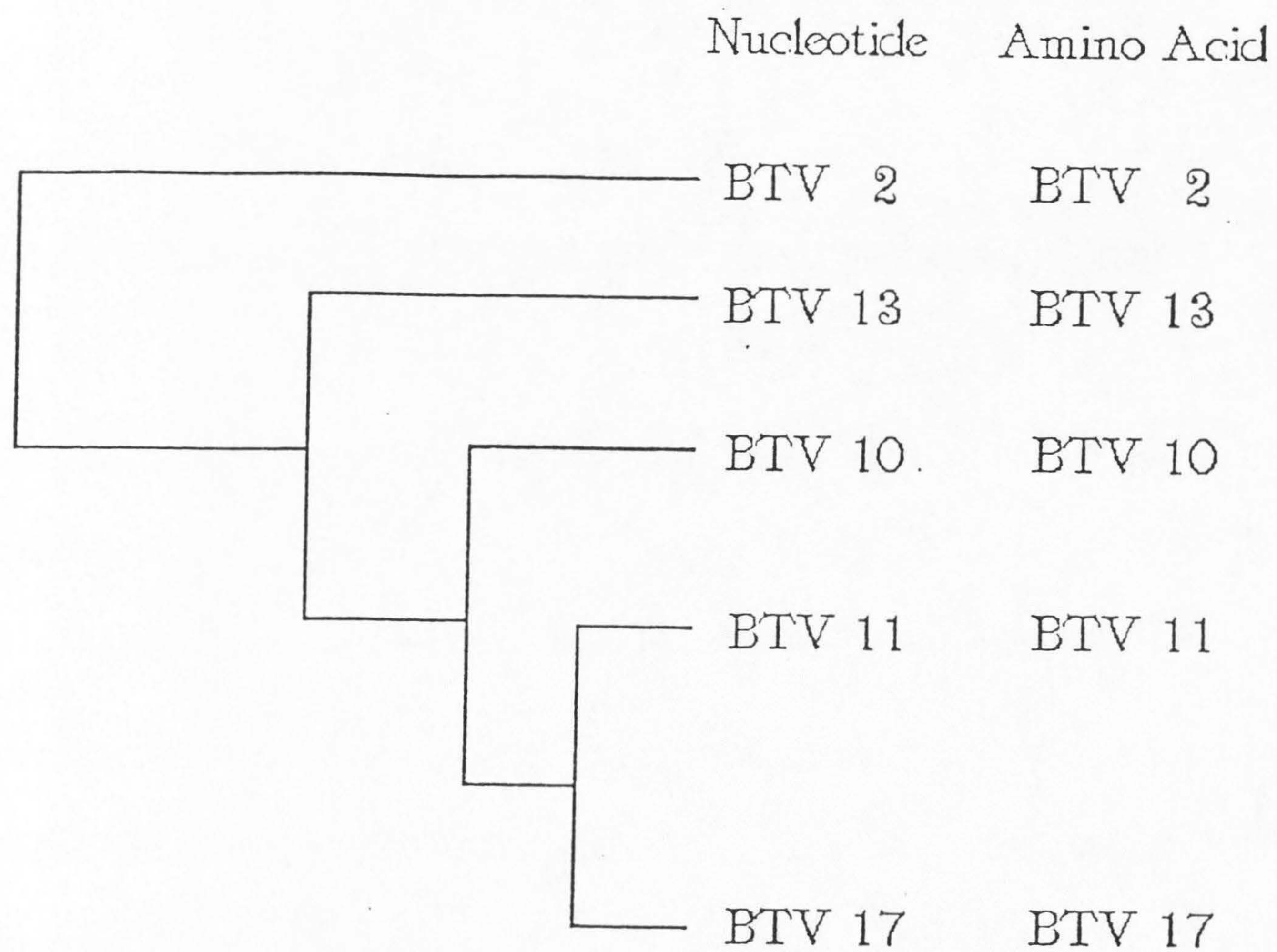


FIG. 4. Results of phylogenetic analysis of the five US BTV serotypes based on nucleotide and deduced amino acid sequences of M1 (segment 4) genes.

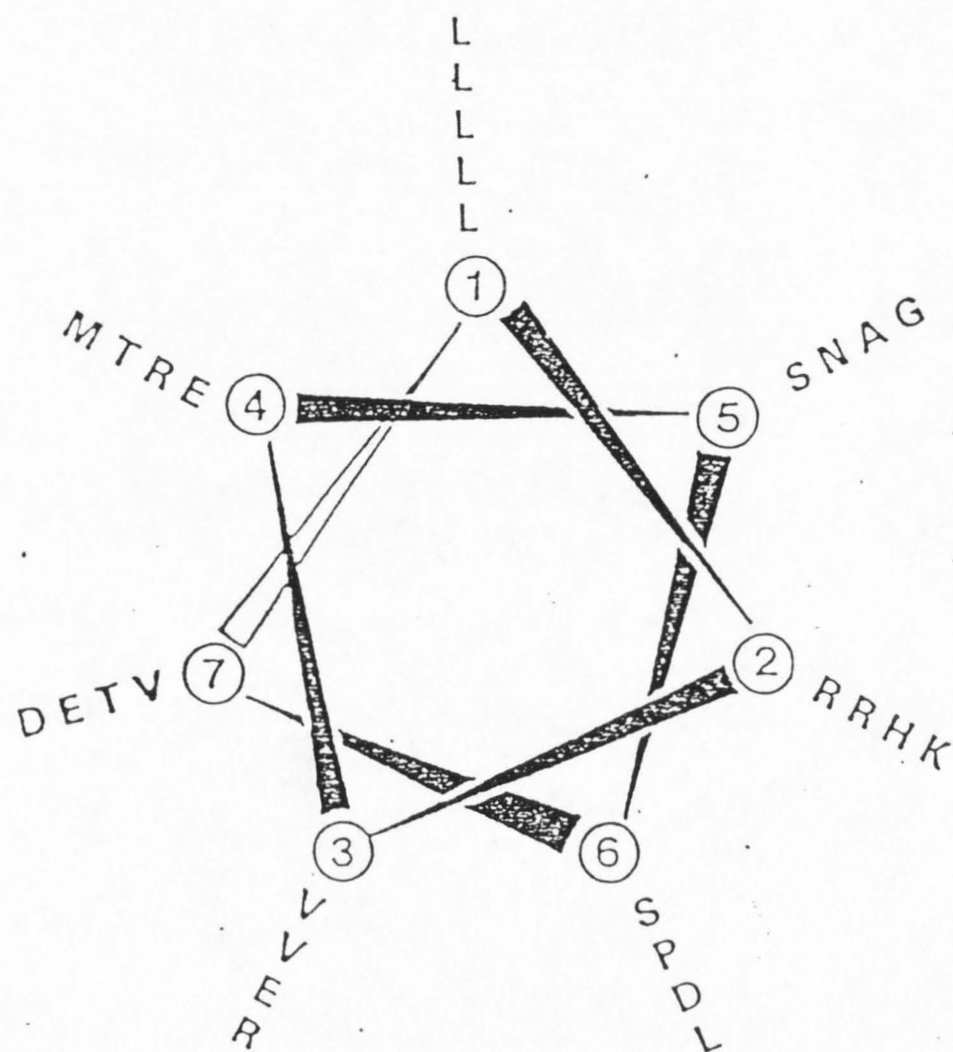


FIG. 5. Helical wheel representation of the leucine repeat found in the potential leucine-zipper motif in the VP4 proteins of five US bluetongue viruses.

CHAPTER III

DELINEATION OF THE ANTIGENIC DETERMINANTS AND SSRNA-BINDING DOMAINS OF THE MULTIMERIC BLUETONGUE VIRUS VP4 PROTEIN

ABSTRACT

Heterologously expressed bluetongue virus (BTV) inner core protein VP4 was purified to near homogeneity. Six oligoclonal antibodies produced in rabbits were able to react with VP4 protein. These six linear epitopes of VP4 were mapped to both termini and the middle of the protein. Enzyme-linked immunosorbent assay (ELISA) and peptide competition assay demonstrated that these six linear epitopes are surface accessible. VP4 was shown to be a ssRNA-binding protein, and the leucine-zipper motif of VP4 was not required for the RNA-binding activity. VP4 protein exhibited similar binding activity to both capped and non-capped mRNAs. Its RNA-binding activity was not specific to BTV ssRNA since VP4 protein could bind to poly(U), and its BTV ssRNA-binding activity could be competed out in the presence of yeast tRNA in a reaction mixture. VP4 was also shown to be an oligomer by chemical cross-linking VP4 that was produced by *in vitro* translation. One RNA-binding domain was mapped by Northwestern assay and deletion mutant

analyses between amino acid residues #112-158. Using sequence specific synthetic peptides corresponding to the arginine- and lysine-rich regions of VP4, four other potential ssRNA-binding domains of VP4 were identified. Although BTV mRNA was assumed to be capped, I found that both capped and non-capped BTV mRNAs synthesized *in vitro* were translated with similar efficiency by a wheat germ extract *in vitro* translation system.

INTRODUCTION

Bluetongue virus (BTV), an agent that causes bluetongue disease in domestic animals and wild ruminants, has been known for more than a century. It is classified in the *Orbivirus* genus of the family *Reoviridae* (Borden *et al.*, 1971; Murphy *et al.*, 1971). The icosahedral-shaped BTV has a double layered protein coat, and its genome is composed of 10 segmented dsRNAs (Verwoerd *et al.*, 1972). There are two outer capsid proteins (VP2 and VP5) and five inner capsid proteins (VP1, VP3, VP4, VP6, and VP7) in the double-layer viral protein coat.

The three minor inner core proteins (VP1, VP4, and VP6) were suggested to be the viral RNA-dependent RNA polymerase and the capping enzymes of BTV (Van Dijk and Huismans, 1980; Urakawa and Roy, 1989; Le Blois *et al.*,

1992). Recombinant VP1 expressed in insect cells had RNA-dependent RNA polymerase activity in the presence of poly(U) templates (Urakawa and Roy, 1989). VP6 has been shown to have nucleic acid binding activities, and its nucleic acid binding domains were identified by Hayama and Li (1994). However, the biological function(s) of VP6 is still unknown. VP4 prepared from both purified virus and recombinant proteins has a GTP-binding activity (Le Blois *et al.*, 1992). It is the prime candidate for being the guanylyltransferase of BTV. However, the capping activity of VP4 has never been proven.

The M1 genes coding for the VP4 proteins of five US BTV serotypes were cloned and sequenced (Chapter II). The sequences of all five VP4 proeins have a very high degree of homology (96-99%) and conserved hydrophilic and hydrophobic regions, determined from hydropathy plot analyses. From the deduced amino acid sequences of VP4, a unique leucine-zipper motif and several basic amino acid clusters were found in this protein. These motifs are found in many nucleic acid binding proteins (Perutz, 1992). Whether VP4 is a nucleic acid binding protein is unknown.

In this study, I intend to characterize the structure of VP4 as well as to investigate the function(s) of VP4.

Using recombinant VP4 proteins, VP4 was shown as an RNA-binding protein and its structure was characterized.

MATERIALS AND METHODS

Construction of recombinant plasmids

The full-length BTV-13 M1 gene in the plasmid pUC19BTV13M1 (Chapter II) was subcloned into the Pst I site of a baculovirus expression vector pVL1392 (Invitrogen). The pUC19BTV13M1 plasmid was digested with Pst I and resolved on a 1% low-melting agarose gel. After electrophoresis, the band corresponding to the size of M1 gene was excised from the gel, the gel slice was melted and ligated to Pst I-digested and CIP-treated pVL1392 expression vector, as described in Chapter II. The orientation of the recombinant plasmid carrying BTV-13 M1 gene was confirmed by BamH I restriction enzyme digestion. The recombinant plasmid for transfection into insect cells was purified according to Kraft *et al.* (1988) and further purified by polyethylene glycol (PEG) precipitation. To produce BTV-13 M1 mRNA for *in vitro* transcription and translation, the BTV-13 M1 gene was cloned into pGEM4Z (pGEM4ZBTV13M1) using the same procedures. A mutant, in which the leucine-zipper region was deleted, was

constructed by digesting pGEM4ZBTV13M1 with Xba I. This enzyme cut at nucleotide positions #1409 and #1734 inside the BTV-13 M1 cDNA, and at nucleotide #34 inside the pGEM4Z vector. After digestion, the reaction mixture was resolved on a 1% low-melting point agarose gel. Two fragments without the leucine-zipper coding region (1.4 kb and 3 kb) were excised from the gel and ligated to each other. The orientation of the insert was checked by Kpn I restriction enzyme digestion. This leucine-zipper deleted mutant had an internal deletion between bases #1409-1733 (amino acid residues #468-575), and was in-frame to the C-terminal coding region.

Construction of recombinant plasmids and 3′-deletion mutants for expression in *E. coli*

The full-length cDNA of the BTV-13 M1 gene was amplified from the dsRNA genome of BTV according to the method described in Chapter II. A pair of primers containing the sequences of 5′-GACGTCGACCCGGGG<u>ATGCCTGAGCCA</u>-3′and 5′-GACGTCGACCCGG<u>GTAAGTTGTACAT</u>-3′ (the sequences of the BTV M1 gene are underlined) was used for amplification, and the cDNA was cloned into the pTrxFus (Invitrogen) expression vector at the Sma I site. This enzyme removed the 5′ non-coding region (eight bases), and fused the

remaining M1 cDNA in-frame to the region coding for thioredoxin. In this expression system, the target proteins are expressed as thioredoxin (Trx) fusion proteins. The recombinant plasmids were transformed into *E. coli* GI698 or GI724 (Invitrogen) by electroporation (Gene Pulser™, Bio-Rad, settings of 25 µF, 2.5 kV, 200 Ω, 4 msec). The orientation of the insert was determined by BamH I enzyme digestion.

The pTrxBTV13 plasmid was used for constructing the 3′ deletion mutants of the M1 gene with the Erase-A-Base kit (Promega). Because exonuclease III (Exo III) will digest the nicked DNA, supercoiled plasmids are required to construct deletion mutants. To purify supercoiled plasmids, acidic phenol extraction was used. An overnight *E. coli* culture (200 ml) was centrifuged at 2000x g for 10 min. The *E. coli* pellet was resuspended in 10 ml of lysis buffer (25 mM Tris-HCl, pH 8, 50 mM glucose, 10 mM EDTA, 5 mg/ml lysozyme) and incubated at room temperature for 5 min. A total of 20 ml 0.2 N NaOH and 1% SDS mixture was then added to the solution and incubated on ice. After 10 min incubation, 12.5 ml of saturated ammonium acetate was added to the solution, and gently mixed for 30 sec, followed by an additional 10 min incubation on ice. The solution was then centrifuged at 12,000x g for 10 min at

4° C. The supernatant was passed through a glass-wool plugged funnel into a centrifuge bottle. Seven-tenths volume of ice-cold isopropanol was added to the filtrate, mixed, and kept on ice for 20 min. DNA was recovered by centrifugation at 12,000x g for 15 min at 4° C. The DNA pellet was then dissolved in 6 ml of acidic extraction solution (0.75 M NaCl, 10 mM EDTA, 0.3 M sodium acetate, pH 4.2), and kept on ice for 5 min. An equal volume of water-saturated phenol was added to the DNA solution, vortexed for 1 min, and centrifuged at 12,000x g for 10 min at 4° C. The top layer was discarded and the acidic extraction was repeated once. The organic phase was then mixed with 3.5 ml of reverse extraction buffer (1.5 M Tris-base, 5 mM EDTA) and 3.5 ml of chloroform. After centrifugation, the top layer containing the supercoiled plasmids was removed to a new tube and extracted once with an equal volume of chloroform. The supercoiled plasmids were then precipitated by addition of 0.1x volume of 3 M sodium acetate, pH 4.2, and 0.7x volume of ice-cold isopropanol. The plasmids were recovered by centrifugation at 4,000 rpm for 15 min at 4° C and washed twice with 70% ice-cold ethanol. The super-coiled plasmid DNA pellet was then dried and dissolved in TE.

To construct the deletion mutants, the supercoiled plasmids were digested with Pst I and Nco I restriction enzymes to create an Exo III (Promega) resistant 3'-overhang end (within the expression vector) and a 5'-unprotected end (3'-end of the M1 gene) in the linearized plasmids. The linearized plasmids were analyzed by gel electrophoresis to confirm that digestion was completed, then purified by phenol/chloroform extraction as described in Chapter II. The linearized plasmids (5 µg) were dissolved in 60 µl of Exo III buffer (66 mM Tris-HCl, pH 8.0, 0.66 mM $MgCl_2$) and then digested with 300 units of Exo III at 15° C. At every 30-sec interval, 2.5 µl of the reaction mixture were transferred to a tube containing 7.5 µl of S1 nuclease mixture (2 units S1 nuclease, 40 mM potassium acetate, pH 4.6, 333 mM NaCl, 1.25 mM $ZnSO_4$, 6.75% glycerol) and kept on ice to stop the Exo III digestion. At the end of sample collection, the DNA samples were incubated at room temperature for 30 min to start the S1 nuclease digestion, to remove the single-stranded tails left after the Exo III digestion. One µl of S1 nuclease stop buffer (0.3 M Tris base, pH 10, 0.05 M EDTA) was added, followed by heating at 70° C for 10 min to inactivate the S1 nuclease. One µl of Klenow mixture (100 u/ml of Klenow DNA polymerase, 20 mM Tris-HCl, pH 8.0, 100

mM $MgCl_2$) was added to the reaction mixture and incubated for 5 min at 37° C, before 1 µl of dNTP mixture (0.125 mM each of dATP, dCTP, dTTP, dGTP) was added to generate blunt ends for ligation. The ligation mixtures were then used to transform *E. coli* strains GI698 and GI724, and the transformed *E. coli* was selected on agar plates with ampicillin. The deletions were confirmed by DNA sequencing, and expression of the truncated proteins was confirmed by immunodetection with antibodies.

Expression and purification of VP4 in the recombinant baculovirus system

Insect cells were grown and maintained as described by Johnson and Li (1991). Co-transfection with the recombinant pVL1392BTV13M1 plasmid and linear baculoviral DNA (Baculogold™) was performed according to the manufacturer's protocol (PharMingen). A monolayer of SF-9 cells grown in Grace's medium with 10% fetal bovine serum or High-5™ cells grown in serum-free Excel 405 medium (J.R. Scientific) at 80% confluence were infected with recombinant baculovirus carrying the BTV-13 M1 gene at a multiplicity of infection (M.O.I.) of 10 to 20. After 72 hr postinfection, the insect cells were centrifuged (Beckman GPR Centrifuge) at 1,000 rpm for 10 min, and the

cell pellets were washed with phosphate-buffered saline (PBS). The cell pellets were resuspended in STE (10 mM Tris-HCl, pH 8, 1 mM EDTA, and 150 mM NaCl) and lysed by sonication (VirSonic, 80% power output) three times (10 sec each) at 0° C. Recombinant VP4 was identified by comparing the cell lysate to mock-infected cells with both SDS-PAGE and immunodetection. VP4 was found mainly in insoluble form due to aggregation. The insoluble VP4 was pelleted by centrifugation at 4,000 rpm for 15 min at 4° C. Because of the high yield of VP4, a major portion of the pellets was composed of VP4. Insoluble VP4 was dissolved at a final concentration of 0.3 mg/ml in STE containing 5 mM dithiothreitol (DTT), 0.15% N-lauroyl sarcosine, and 0.01% Triton X-100 (TX-100). After 30 min of incubation at room temperature, insoluble materials were removed by centrifugation at 4,000 rpm for 30 min. The soluble VP4 in the supernatant was then dialyzed against STE with 0.05% N-lauroyl sarcosine, 0.01% TX-100, and 10 mM reduced glutathione for 5 hr at 4° C. The dialysis buffer was then changed to STE with 0.01% TX-100 and 5 mM reduced glutathione and dialyzed overnight at 4° C. The dialyzed protein solution was then centrifuged at 4,000 rpm for 30 min at 4° C to remove any aggregated protein. The dialysate was passed once through a Sephadex G-75

(Pharmacia) sizing column pre-equilibrated with STE. The soluble VP4 was found in the void volume and the low molecular weight cellular proteins were retained in the column. VP4 was further purified through a 5 to 50% (w/v) discontinuous sucrose gradient with a cushion of 66% sucrose (w/w) in STE. After centrifugation at 30,000 rpm (Beckman L8-70 Ultracentrifuge) for 1.5 hr at 4° C in a SW41 rotor, VP4 was located at the interface of the 50% and 66% sucrose solution.

Expression of VP4 in *E. coli*

The full-length and 3'-end deleted M1 genes cloned into the expression vector pTrxFus were transformed into *E. coli* strain GI698 or GI724 by electroporation as previously described. The expression procedures from the manufacturer (Invitrogen) were followed. An overnight culture of *E.coli* in RM medium (1x M9 salt, 2% casamino acid, 1% glycerol, 1mM $MgCl_2$, 100 µg/ml ampicillin) was grown at 30° C and diluted 20-fold with the induction medium (1x M9 salt, 0.2% casamino acid, 0.5% sucrose, 1mM $MgCl_2$, 100 µg/ml ampicillin). After 2.5 hr of shaking at 30° C, tryptophan was added to the culture at a final concentration of 100 µg/ml as an inducer. The temperatures for VP4 expression in *E. coli* GI698 and GI724 were 30° C and 37° C,

respectively. At 3.5 hr postinduction, the *E. coli* cells were harvested, lysed, and analyzed by gel electrophoresis and by immunodetection.

Production of oligoclonal and monoclonal antibodies

The locations of potential antigenic epitopes of VP4 were predicted using the PlotStructure Program in the GCG software (Genetic Computer Group, 1994). Six synthetic peptides synthesized in the multiple antigen peptide format were used as immunogens to produce oligoclonal antibodies (OAbs) against VP4 (Li and Yang, 1990; Yang *et al.*, 1992; Huang *et al.*, 1995). The positions of these six peptides were residues: #617-626 (RALREYKRKM), #583-592 (KEQKLRDLKR), #167-178 (GARFDDEPTDEK), #376-385 (RDRGTFLKKR), #103-115 (ENEIGRRRIRMRK), and #523-551 (RVESSVLRVRNPTLHETADELKRMGLDL). The corresponding OAbs are designated as OAb #76, 77, 78, 80, 81, and 82, respectively. Rabbits were immunized intramuscularly with 0.5 ml of the peptide solution (100 µg of each individual peptide resuspended in PBS) mixed with an equal volume of Complete Freund's Adjuvant (Sigma). Booster injections followed every 3 weeks with a mixture of antigen and Incomplete Freund's Adjuvant (Sigma). Between each booster injection, 10 ml of sera were collected from the rabbits

for analysis of the antibody titers. After the fourth booster, rabbits were bled, and the antisera were collected and pooled for immunodetections. Recombinant VP4 expressed in insect cells was used as an antigen to produce anti-VP4 monoclonal antibodies (MAbs) by the Biotechnology Center at Utah State University. The anti-VP4 MAbs (D3.6.24 and D3.10.1) were determined to be the IgM isotype.

Enzyme-linked immunosorbent assay (ELISA)

ELISA using OAbs and MAbs was carried out as described by Yang *et al.* (1992) with some modifications. Purified VP4 proeins (5 µg) in 50 µl of 10 mM sodium carbonate, pH 9.6, were immobilized overnight at 4° C to the bottom of a 96-well plate (Dynatech). The plate was washed six times with PBS containing 0.05% Tween-20 (Sigma) (PBS-T), and blocked with 200 µl of 3% bovine serum albumin (BSA) in PBS-T for 1 hr at 37° C. The plate was washed with PBS-T again before antibodies were added to the wells. The antibodies were diluted at 1:10, 1:100; 1:1,000, and 1:10,000 in PBS-T for the assay. After 90 min of incubation at room temperature, the plate was washed four times with PBS-T to remove the unbound antibodies. Second antibodies (alkaline phosphatase(AP)-conjugated goat anti-rabbit IgG or AP-conjugated goat anti-mouse IgM antibodies)

were added to the wells at 1:10,000 dilution and incubated at 37° C for 1 hr. The plate was then washed with PBS-T 4 times and another four times with AP developing buffer (100 mM Tris-HCl, pH 9.5, 5 mM $MgCl_2$, 100 mM NaCl). The color reaction was developed with the addition of AP substrate, p-nitrophenyl phosphate, disodium (pNPP) (Sigma) in the AP developing buffer mixture. The extent of binding was measured in triplicate at 405 nm by an ELISA reader (Dynatech).

SDS-PAGE and immunodetection

The protocols used for SDS-PAGE and immunodetection were similar to those routinely employed in our laboratory. Briefly, proteins were separated on a discontinuous SDS-PAGE using the method of Laemmli (1970) and transferred to two nitrocellulose membranes by a diffusional sandwich technique, in buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% (v/v) methanol. The epitope analyses by immunodetection were carried out as previously described by Li and Yang (1990).

Immunoblocking assay by synthetic peptides

Each of the synthetic peptides (50 µg) dissolved in PBS was mixed with 20 µl of ascites fluid and incubated for

2 hr with constant agitation at 37° C. The MAb incubated with PBS only (without any peptide) was used as a positive control. After a brief centrifugation, the cross-absorbed MAbs in the supernatant were used for immunodetection (1:400 dilution) against recombinant VP4 in SF-9 cell lysate in Western blot analyses. The loss of reactivity with VP4 indicated the possible location of the epitope.

In vitro transcription and translation of mRNA of M1 gene

The pGEM4Z vectors carrying the full-length BTV-13 M1 gene or the leucine-zipper deleted mutant were linearized with Sma I, and RNA transcripts were synthesized using T7 RNA polymerase. The reactions were carried out in the presence or absence of an RNA-cap analog (BRL). For making non-capped mRNA, 6 µg of linearalized plasmids were incubated in a final volume of 100 µl reaction mixture containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM of each rNTP, and 50 units of T7 RNA polymerase at 40° C for 1.5 hr. For making capped mRNA, 0.5 mM of RNA cap analog (BRL) and 0.05 mM rGTP were used to replace the 0.5 mM rGTP in the reaction mixture. The synthesized mRNA should have more than 90% capped mRNA using the *in vitro* transcription kit with RNA

cap analog (BRL), according to the manufacturer. At the end of the reaction, the DNA templates were digested with DNase (BMB) and the RNA transcripts were extracted with phenol-chloroform and precipitated with ethanol. An amount of transcript (0.3 µg) dissolved in H_2O was used for *in vitro* translation with 25 µl of wheat germ extract (Promega), 4 µl of 1mM amino acids mixture (without methionine) (Promega), 1 µl of RNase inhibitor (100u/µl) (USB), and 2.5 µl of [^{35}S]-methionine (10 mCi/ml) (Amersham) in a 50-µl reaction mixture, at 25° C for 2 hr. Before mixing the mRNA with the translation mixture, the template mRNA was heated at 67° C for 10 min and chilled on ice immediately to destroy local regions of secondary structure. The amount of mRNA used for *in vitro* translation was optimized and found to be 0.06 µg per 10 µl of reaction. The translation mixtures were centrifuged for 5 min at 4° C in a microcentrifuge before subsequent analyses.

Poly(U)-Sepharose 4B affinity chromatography

Ten µl of the *in vitro* translated intact or truncated VP4 proteins were diluted with 200 µl of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) and applied onto a 0.6 ml poly(U)-Sepharose 4B column (Sigma) pre-equilibrated with TE. The column was then washed with TE until no radioactivity was

detected in the flow-through fractions, monitored with a GM counter. The bound VP4 proteins were eluted with a step gradient of 0.15, 0.5, and 1.5 M NaCl in TE (pH 8). The eluants were precipitated by 10% trichloroacetic acid (TCA), with 10 µg of bovine serum albumin (BSA) as a carrier, at 0° C for 20 min. After centrifugation at 10,000x g for 10 min, the pellets were washed with 70% ethanol or acetone and dried at 37° C. The pellets were dissolved in 15 µl of SDS-PAGE sample buffer and boiled for 3 min before SDS-PAGE analysis. Pre-stained protein markers were used as molecular weight references. At the end of electrophoresis, the gels were dried under vacuum at 80° C and exposed to X-ray film for autoradiography.

RNA probes preparation

Both capped and non-capped [^{32}P]-labeled BTV M1 ssRNAs were *in vitro* transcribed as described above except that [α-^{32}P]-rGTP (15 µCi/ml) was included in the reaction mixture. Nonradioactive RNA probes were labeled with biotin (BRL).

Electrophoretic mobility shift assay (EMSA)

Secondary structures of the ssRNA were initially disrupted by incubating at 70° C for 10 min and chilling on

ice immediately. Various amounts of purified VP4 in STE were incubated with 28 ng of [^{32}P]-ssRNA at room temperature (about 22° C) for 20 min in a reaction buffer containing 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 60 mM NaCl. The protein-RNA complexes were resolved on a 0.8% agarose gel (0.5x TBE buffer) at 100 V until the bromophenol blue dye front reached the bottom of the agarose gel. The gels were fixed with 10% methanol and 10% acetic acid for 15 min and dried between two sheets of BioGel wrap membranes (BioDesign) at 37° C prior to autoradiography.

Northwestern Assay

Various amounts of sequence-specific peptides were dot-blotted to a nitrocellulose membrane. The membrane was then washed with 0.1x STE buffer and incubated with [^{32}P]-ssRNA for 30 min. The membrane was washed with the same buffer three times (10 min each), air dried, and used for autoradiography.

To develop the Northwestern assay using a biotinylated RNA probe, the nitrocellulose membrane was first blocked with 3% BSA in binding buffer for 1 hr, followed by adding the RNA probes to the binding solution and incubating for 30 min. The bound RNA probes were detected with avidin-conjugated horseradish peroxidase (HRP). The color

reaction was developed in 10 ml of developing solution (0.6 mg/ml of diaminobendizine in 9 mM Tris-HCl, 0.03% $CoCl_2$, and 0.03% H_2O_2).

For the mapping of RNA-binding domain using truncated Trx-VP4 fusion proteins, proteins were separated on SDS-PAGE and transferred overnight at room temperature in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) to nitrocellulose membranes (Li and Yang, 1990). The proteins were allowed to "renature" by washing membranes in binding buffer (10 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA, and 1x Denhardt's solution) at room temperature for at least 1 hr with three changes of buffer. [^{32}P]-labeled RNA probes were added to 10 ml binding buffer and incubated with the nitrocellulose membrane containing the transferred proteins at room temperature for 1 hr. The membrane was then washed with binding buffer three times for 5 min each. The membrane was then air dried and exposed to X-ray film for autoradiography.

Chemical cross-linking of VP4 protein

In vitro translated VP4 proteins (3 µl) were diluted with 87 µl of cross-linking buffer (20 mM HEPES, pH 8, 150 mM NaCl, 1 mM EDTA) to a final volume of 90 µl. Cross-linking was initiated by the addition of 10 µl of 0.1%

glutaraldehyde (50% EM grade, Sigma). At predetermined times of incubation at room temperature, the cross-linking reactions were terminated with the addition of 100 µl of 1 M glycine. Proteins were precipitated with TCA (10%) at 0° C for 20 min with 10 µg of BSA as a carrier. Precipitants were washed with cold acetone were resuspended in SDS-PAGE sample buffer, then resolved on a 3.5% SDS-PAGE (1.5 mm thickness) gel prepared according to Sigma Technical Bulletin (MWS-877). Proteins were separated by electrophoresis at 50 mA (room temperature) for 13 hr. At the end of electrophoresis, the gel was stained by Coomassie blue and destained to visualize and locate the molecular weight markers. The gel was then dried under vacuum (80° C) and used for autoradiography. Cross-linked phosphorylase b proteins (Sigma) were used as molecular weight markers.

RESULTS

Production and purification of recombinant VP4 in insect cells

SF-9 or High-5 cells infected with recombinant baculovirus carrying the BTV-13 M1 gene expressed high levels of VP4. The recombinant VP4 had a molecular weight close to 75 kDa as determined by SDS-PAGE. This molecular

weight is similar to that predicted from the deduced amino acid sequence (Huang *et al.*, 1993) and to the VP4 in purified BTV virions. The level of VP4 expression was M.O.I.-dependent (Lane 1 and 2, Fig. 6A). The identity of the expressed VP4 was confirmed on a Western blot by immunodetection using a polyclonal antibody against BTV virions (Fig. 6B and C). The bulk of recombinant VP4 expressed in insect cells was insoluble even though VP4 has a high content of charged and polar amino acid residues (Huang *et al.*, 1993). This was also true when VP4 was expressed as thioredoxin (Trx)-VP4 (Invitrogen), glutathione-S-transferase (GST)-VP4 (Pharmicia), and six histidines (6xH)-VP4 (Qiagen) fusion proteins in different strains of *E. coli* and under different induction conditions (data not shown). Many conditions were employed in the attempt to purify a large quantity of VP4 for structure and function analyses. Unfortunately, no method worked to produce soluble VP4. These methods, which are summarized in Appendix D, have been tried and investigated without success. However, the insoluble VP4 was solubilized successfully upon treatment with STE buffer containing 0.15% N-lauroyl sarcosine, 5mM DTT, and 0.01% TX-100. The detergents were removed by stepwise dialysis, which allowed the VP4 protein to be maintained in its soluble form. The

optimal VP4 concentration for refolding and renaturation was 0.3 mg/ml. Concentrations higher than 0.3 mg/ml resulted in rapid precipitation to an insoluble form. VP4 was further purified to near homogeneity by a sizing column and sucrose gradient (Fig. 7). Because VP4 formed an octameric structure at a molecular weight of 600 kDa (see below), the VP4 oligomer was in the void volume during elution from the Sephadex G-75 sizing column. The VP4 oligomers could be separated from other proteins by discontinuous sucrose gradients. The VP4 oligomers were found at the interface of 50% (w/v) and 66% (w/w) sucrose solutions after ultra-centrifugation at 110,000x g for 1.5 hr.

Identification of the antigenic epitopes of VP4

To identify and map the linear antigenic epitopes of VP4, potential antigenic epitopes of the cognate VP4 were predicted by the GCG computer package. Six oligopeptides were synthesized in the multiple antigen peptide (MAP) format (Tam, 1988; Li and Yang, 1990; Yang *et al.*, 1992 Huang *et al.*, 1995) and then used to produce six OAbs (OAb #76, 77, 78, 80, 81, and 82) in rabbits. The OAbs were analyzed by immunodetection on Western blots (Fig. 8) and ELISA (Fig. 9). In Western blot immunodetection as shown in Fig. 8, OAb #78, 81, and 82 reacted well with VP4 at

1:1,000 dilution; OAb #80 had a moderate reactivity at 1:500 dilution; OAb #76 and 77 had a very limited reactivity even at 1:250 dilution. These results are evidence that amino acid residues between #617-626 (RALREYK RKM), #583-592 (KEQKLRDLKR), #167-178 (GARFDDEPTDEK), #376-385 (RDRGTFLKKR), #103-115 (ENEIGRRRIRMRK), and #523-551 (RVESSVLRVRNPTLHETADELKRMGLDL) represent linear epitopes or major linear components of conformational epitopes of VP4.

To determine whether these antigenic epitopes are surface accessible, an ELISA was carried out using these six OAbs against purified VP4 (Fig. 9). In ELISA tests, OAbs #76, 78, 81, and 82 had strong reactions with the native and intact VP4, and OAb #77 and 80 had weaker reactivities. These results are evidence that the antigenic domains corresponding to the sequences of peptides #76, 77, 78, 80, 81, and 82 are potential linear epitopes on the surface of VP4. OAb #76 had a very weak reactivity against the denatured VP4 on a Western blot (Fig. 8), but had a strong reactivity against native VP4 in ELISA (Fig. 9). This implied that the conformation of this region is important for the reaction with OAb #76.

Monoclonal antibodies against VP4 (D3.6.24 and D3.10.1) were produced for an attempt to map conformational epitopes (Fig. 9 and 10). To identify the location of

these conformational epitopes, an epitope-scanning approach (Hwang and Li, 1993) with sequence-specific synthetic peptides was used to block binding of MAbs towards VP4 on a Western blot (Fig. 10). The two MAbs in ascites fluid were incubated with 50 µg of each synthetic peptide (#76, 77, 78, 80, and 81) individually at 37° C for 2 hr with constant agitation. The cross-absorbed ascites fluids were then used for immunodetection on a Western blot (Fig. 10). As shown in Fig. 10A, peptides #76, 80, and 81 blocked the binding of MAb D3.6.24 to VP4. Since the locations of peptides #76 (residues #617-626), #80 (residues #376-385), and #81 (residues #103-115) are located in different regions of VP4, this suggests that the antigenic epitope defined by MAb D3.6.24 is a conformational determinant. As shown in Fig. 10B, only peptides #76 (residues #617-626), and #81 (residues #103-115) blocked the binding of MAb D3.10.1 to VP4. These results are evidence that the epitope recognized by MAb D3.10.1 is conformational and were also consistent with the results of ELISA that peptides #76, 80, and 81 are surface accessible.

Nucleic acid binding activity of VP4

VP4, a GTP-binding protein, is a candidate for being the guanylyltransferase of BTV (Le Blois *et al.*, 1992). A

potential leucine-zipper motif and a stretch of charged amino acid residues upstream of the zipper region were identified from the deduced amino acid sequences of five US BTV VP4 proteins (Huang *et al.*, 1993). These features are found in many nucleic acid binding proteins. The proposed guanylyltransferase activity of VP4 suggested that this protein may be a ssRNA-binding protein. Therefore, the ssRNA-binding activity of VP4 was investigated.

As shown in Fig. 11A, the binding of VP4 to the BTV ssRNA resulted in retardation of the migration of the labeled RNA transcripts in gel electrophoresis. The amount of RNA retarded in EMSA was proportional to the amount of VP4 added to the reaction mixture. When non-labeled, non-capped RNA transcripts were added to the RNA-binding reaction mixture, the amount of labeled, retarded RNA transcripts was inversely proportional to the amount of added non-labeled RNA. This is evidence that the binding of VP4 to ssRNA was not dependent on the cap structure of the transcript (Fig. 11B). The specificity of VP4 ssRNA-binding activity was also determined by competition with unlabeled yeast tRNA. As shown in Fig. 12, the labeled ssRNA transcripts were not retarded when 500 µg of unlabeled yeast tRNA was added to the reaction mixture. Upon the addition of 1% SDS to the reaction mixture, VP4

aborted the RNA-binding activity (Lane 5, Fig. 12). This is evidence that the nondenatured form of VP4 is required for RNA-binding. The identity of RNA was confirmed by incubating the RNA with RNase A in 60 mM NaCl. The ssRNA was digested by this nuclease treatment (Lane 6, Fig. 12).

Because the leucine-zipper motif is involved in nucleic acid binding activity of some proteins (Perutz, 1992), the role of the VP4 leucine-zipper motif in the RNA-binding activity was investigated. The intact and the truncated VP4 (VP4ΔLZ), which has an internal deletion of the leucine-zipper region, were translated *in vitro* (Fig. 13B), and their RNA-binding activity toward poly(U)-sepharose was tested.

In vitro translated VP4 was able to bind to poly(U)-sepharose (Fig. 3A). VP4 was eluted with 0.15 M NaCl in TE at pH 8. The deletion mutant without the leucine-zipper motif was also able to bind to the poly(U) in the sepharose column (Fig. 14B). These results are evidence that the leucine-zipper motif within the VP4 protein is not required for the RNA-binding activity.

Mapping the ssRNA-binding domains of VP4

To locate the RNA-binding domain of VP4, a series of C-terminus deleted VP4 proteins were constructed by

deleting the 3'-end of the M1 gene in the pTrxVP4 plasmid (see MATERIALS AND METHODS). These C-terminal truncated VP4 proteins were expressed as thioredoxin (Trx) fusion proteins. Trx-VP4 and most of the truncated Trx-VP4 mutants were expressed as an insoluble form. Nucleic acid binding on protein blots has been used to identify DNA- (Bowne *et al.*, 1980; Hoch, 1982) and RNA- (McCormack *et al.*, 1992; Labbé *et al.*, 1994) binding proteins. Since it is difficult to purify the insoluble Trx-VP4 truncated protein for the RNA-binding assay in EMSA, I used a similar technique to detect the RNA-binding domain of VP4 on protein blots (Northwestern assay). To determine whether the VP4 expressed in *E. coli* retained RNA-binding activity, a Northwestern assay was carried out using VP4 expressed both in insect cells and in *E. coli* (Trx-VP4). The VP4 expressed in insect cells was used as a positive control. As shown in Fig. 15, the VP4 proteins expressed in both systems have ssRNA-binding activity. The RNA-binding efficiency of VP4 in the Northwestern assay was not equivalent to the amount of protein on the membrane, but might result from different renaturing efficiencies of VP4 on membranes. The ssRNA-binding capability of VP4 on the protein blot was also sensitive to 0.15 M NaCl. When an RNA-bound protein blot was washed with TBST (10 mM Tris-

HCl, pH 8, 150 mM NaCl, 0.3% Tween-20), the bound RNA was released from the protein (data not shown). This result corresponded to that obtained from the poly(U)-sepharose column (Fig. 14). The same technique was also used to map the RNA-binding domains on protein blots with Trx-VP4 and truncated VP4 proteins. The identities of Trx-VP4 and the truncated VP4 proteins were confirmed by anti-VP4 OAbs (Fig. 16).

The truncated mutants Δ1B and Δ58, which have only amino acid residues #1-111 and #1-76, respectively, lack ssRNA-binding activity (Fig. 17). In contrast, the truncated mutant Δ81, which has amino acid residues #1-158, and all other mutants were able to bind to labeled ssRNA. An interpretation is that a ssRNA-binding domain is located between amino acid residues #112-158. To determine if residues #112-158 represent the only ssRNA-binding domain, an N-terminal truncated Trx-VP4 was constructed (mutant Δ67), which deleted amino acid residues #1-298. The identity of mutant Δ67 was confirmed by OAb #82. As seen in Fig. 16, mutant Δ67 was not able to react with OAb #81, which recognizes the N-terminus of VP4, but did react with OAb #82, which recognizes the C-terminus of VP4. As seen in Fig. 17, mutant Δ67 retained ssRNA-binding

activity. This result is evidence that there is at least one more ssRNA-binding domain of VP4, in the region between residues #299-644, in addition to the one located in the #112-158 region.

Many studies have shown that RNA-binding domains contain clusters of basic amino acid residues (Merrill *et al.*, 1988; Lazinski *et al.*, 1989; Nagai *et al.*, 1990; Lee *et al.*, 1993; Waksman *et al.*, 1993; Hayama and Li, 1994). Many lysine and arginine clusters are found in VP4, which could function as RNA-binding sites. In the studies of BTV VP6, Hayama and Li (1994) used sequence-specific peptides to map the RNA-binding domains of VP6. A similar technique was used to map other RNA-binding domains of VP4.

Peptides of VP4, 9 to 13 residues in length, were used in EMSA and Northwestern assays to map ssRNA-binding domains. As shown in Fig. 18, peptides #76, 77, 80, and 81 retarded ssRNA in EMSA and bound ssRNA on the membrane in the Northwestern assay. Peptide #77 has a lower binding affinity. Peptide #78 has no ssRNA-binding activity in either the EMSA or Northwestern assay. Therefore, these four regions represented by the peptides are the potential ssRNA-binding domains of VP4. The binding ability of these peptides to dsDNA and dsRNA of the BTV genome was investigated. We found that the same peptides that bind to

ssRNA were able to bind to BTV dsRNA, and none of the peptides was able to bind dsDNA (data not shown). Thus, these peptides have specific binding activity to RNA instead of to any type of nucleic acids.

Translational efficiency of capped and non-capped M1 mRNA

It is assumed, but has not been proven, that BTV mRNAs are capped. I decided to investigate whether the cap structure of the BTV mRNA is required for translation and whether capped and non-capped BTV mRNAs synthesized *in vitro* have different translational efficiencies. Both capped and non-capped BTV-13 M1 mRNAs were transcribed as described in Materials and Methods. These mRNAs were used for *in vitro* translation with wheat germ extracts (Promega). As shown in Fig. 13A, equal amounts of both capped and non-capped BTV M1 mRNA (0.06 µg) in 10 µl of the wheat germ translation mixture had a similar efficiency of translation. This result implies that the cap structure of BTV mRNA is not required for translation (at least not in a wheat germ system). The *in vitro* translated VP4 was soluble, and it was found in the supernatant fraction after centrifugation at 10,000x g for 10 min (data not shown).

Oligomerization of VP4

Leucine-zipper motifs are known to be responsible for protein dimerization (Landschulz *et al.*, 1988) and trimerization (Sorger and Nelson, 1989). Therefore, the possibility that VP4 forms an oligomer through its leucine-zipper was investigated. *In vitro* translated VP4 labeled with [^{35}S]-methionine was incubated with glutaraldehyde for various times. After the chemical cross-linking, the product was resolved by SDS-PAGE and examined by autoradiography. As shown in Fig. 19, in the presence of cross-linker and increased incubation time, the amount of VP4 monomer (75 kDa) decreased and the amount of oligomeric protein increased. By comparison with known size markers, the molecular weight of the VP4 oligomer was estimated to be around 600 kDa. This is evidence that VP4 forms a high molecular weight oligomer, probably an octamer in solution. In the Coomassie blue stained SDS-PAGE that separated the chemically cross-linked products, most of the translation factors were not cross-linked. This is evidence that the cross-linking was specific. Since the oligomerization of Hsp104 of *Saccharomyces cerevisiae* is affected by the presence of ATP (Parsell *et al.*, 1994), and VP4 is a GTP-binding protein (Le Blois *et al.*, 1992), the effect of ribonucleotides (rNTP) on VP4 oligomerization was

investigated. Oligomerization of VP4 was not affected by 1 mM of each individual rNTP in the cross-linking mixture (data not shown).

To investigate whether the leucine-zipper motif is required for oligomerization, a cross-linking experiment was performed using the leucine-zipper deleted mutant (VP4ΔLZ). In this case, another bifunctional cross-linker, dimethyl suberimidate dichloride (DMS), was used for the cross-linking reactions. DMS is an imidoester homobifunctional cross-linker that reacts with primary amines on proteins and has a short spacer arm 11 Å in length. As shown in Fig. 20, when truncated VP4ΔLZ was exposed to increasing concentrations of DMS, the monomer was converted into an oligomer. These results are evidence that VP4ΔZ is oligomeric in solution.

DISCUSSION

Each BTV virion has an estimated 5 to 10 molecules of VP4 inside the inner core (Huismans and Van Dijk, 1990). Although VP4 has been suggested to be the guanylyltransferase of BTV because of its GTP-binding activity (Le Blois *et al.*, 1992), GTP-binding and capping activity have not been demonstrated in purified VP4. No other properties

of this protein have been discussed. In this report, I have expressed and purified recombinant VP4 protein to near homogeneity, identified it as a RNA-binding protein, mapped several RNA-binding domains and antigenic epitopes, and demonstrated that VP4 exists as a high molecular weight oligomer.

Recombinant VP4 expressed in insect cells using the baculovirus expression system was mostly insoluble even though it has a high content of hydrophilic amino acids (Huang *et al.*, 1993). After the cells infected with recombinant baculovirus carrying the M1 gene were lysed by detergents, freeze and thaw cycles, or sonication, only very small amounts of soluble recombinant VP4 were detected by immunodetection (data not shown). The expression of recombinant VP4 in insect cells started at 24 hr postinfection. The expressed VP4 aggregated rapidly and became insoluble immediately after its synthesis (data not shown). Expressing VP4 as a GST-VP4 fusion protein in insect cells failed to increase the amount of soluble VP4 (data not shown). Co-expressing VP4 and another inner minor core protein, VP6, did not increase the production of soluble VP4 (Appendix C). The M1 gene was also cloned into the pHIL-S1 (Invitrogen) yeast (*Pichia pastoris*) expression vector. VP4 expressed in this system was fused with a

secretion signal from acid phosphatase (PHO1). We expected the expressed VP4 to be secreted into the growth media of yeast. However, the expressed VP4 aggregated in the yeast and was never secreted into the growth media (data not shown).

VP4 remained in insoluble aggregates when expressed in different strains of *E. coli* as GST-VP4 and Trx-VP4 fusion proteins. These fusions often increase the solubility of the expressed protein. By expressing VP4 as 6-histidine (6xH) tagged fusion proteins, VP4 was purified in the denatured state using a metal affinity column in the presence of urea (Appendix B). When VP4 was expressed at 15° C, more soluble proteins were produced. However, the yield was reduced tremendously. After deleting 400 amino acid residues from the C-terminus, VP4 was expressed in a soluble form.

I took advantage of the fact that VP4 aggregates to separate it from most of the cellular proteins by centrifugation. By using the anionic detergent N-lauroyl sarcosine in the presence of DTT and TX-100, I was able to solubilize and dissociate the VP4 from the aggregates, and let it refold to soluble form by dialysis. Because VP4 forms an oligomer, I was able to purify it to near

homogeneity using a sizing column and a discontinuous sucrose gradient (Fig. 7).

Six OAbs against VP4 were produced using VP4 peptides predicted by the PlotStructure program of the GCG package. These six OAbs have different reactivities against VP4 in Western blots against denatured VP4 (Fig. 8) and in ELISA against native and soluble VP4 (Fig. 9). The result (Fig. 9) that all of the OAbs were able to bind to VP4 in its native form in ELISA is evidence that these epitopes are located on the surface of the protein. OAb #76 (1:250) had a very weak reaction to denatured VP4 (Fig. 8) but had a strong reactivity to the native VP4 in ELISA (Fig. 9). This is evidence that the conformation of the peptide #76 region (residues #617-626) is important for this antibody to interact. It is difficult to conclude that this region is a conformational epitope, since this region is only 9 amino acid residues in length. The surface accessibility of peptides #76, 80, and 81 was confirmed by peptide competition against two MAbs against VP4 (Fig. 10). Because peptides #76, 80, and 81 were able to block the binding activity of MAb D3.6.24 to VP4, and these regions are located at different locations (residues #617-626, #376-385, and #103-115), I hypothesize that MAb D3.6.24 recognizes a conformational determinant composed of three

regions of the primary structure of VP4. This hypothesis is supported by the ELISA results (Fig. 9) and is consistent with predictions of the PlotStructure program (Fig. 21). All of the peptide regions are probably surface located. The results of peptide blocking (Fig. 10) are evidence that both termini of VP4 and the middle of VP4 might come together in its native state.

This is the first report to show that VP4 is a ssRNA-binding protein. The ability of VP4 to bind ssRNA was demonstrated using EMSA (Fig. 11), binding to poly(U)-sepharose (Fig. 14), and Northwestern assay (Fig. 16). The ssRNA-binding activity of the purified recombinant VP4 could be competed out by unlabeled BTV ssRNA homologously and by yeast tRNA heterologously (Fig. 12). This is evidence that the binding of VP4 to ssRNA is not sequence-specific.

Four potential RNA-binding domains were mapped using truncated Trx-VP4 fusion proteins and sequence-specific peptides for the RNA-binding assay (Fig. 17 and 18). These regions are located at amino acid residues #103-115 (ENEIGRRRIRMRK), #376-385 (RDRGTFLKKR), #583-592 (KEQKLRDLKR), and #617-626 (RALREYKRK). These regions are surface accessible to VP4 (Fig. 9 and 16) and are rich in basic amino acid residues (R and K), which are found in

many RNA-binding proteins (Merrill *et al.*, 1988; Lazinski *et al.*, 1989; Nagai *et al.*, 1990).

Two classes of eukaryotic and prokaryotic RNA-binding proteins have previously been identified. One contains the RNA-recognition motif (RRM), which consists of 80 amino acids with two conserved sequences, RNP-1 and RNP-2, of 8 and 6 amino acids, respectively (Bandziulis *et al.*, 1989; Mattaj, 1989). The other one is classified as the arginine-rich motif (ARM) family (reviewed in Mattaj, 1993). These RNA-binding proteins bind to RNA through short, arginine-rich sequences (4 to 8 arginines in a stretch of 10-20 amino acids). They have been found in some RNA viruses (*tat* and *rev* protein of HIV-1), bacteriophages (the N protein of bacteriophage lambda), and ribosomal proteins (Lazinski *et al.*, 1989). The ARM sequence itself is sufficient for RNA recognition. There is little sequence identity among ARMs, and most of the ARM proteins do not share a common structure. The RNA-binding peptides of VP4 are similar to the ARM family. After three RNA-binding domains were deleted, the truncated VP4 was still able to bind to RNA (Fig. 17). This is evidence that the RNA-binding activity of VP4 dose not require the coexistence of the four RNA-binding domains to be functional. A similar result was found in the BTV VP6

protein. Since there are multiple RNA-binding domains of VP6, only one domain is sufficient for the RNA-binding (Hayama and Li, 1994). This is different in hepatitis delta virus antigen (HDAg), which has two ARMs, and both are required for the RNA-binding activity. Deletion of either one of these ARM sequences resulted in the total loss of the *in vitro* RNA-binding activity of HDAg (Lee *et al.*, 1993). Perhaps, each the different RNA-binding domains of VP4 is involved in different functions, such as RNA capping, encapsidation, transcription, or replication.

One RNA-binding domain of VP4 was mapped between amino acid residues #112-158 that has four amino acid residues (112-RMRK-115) that overlap the RNA-binding peptide #81 (103-ENEIGRRRIRMRK-115) (Fig. 18). However, the truncated mutant #Δ1B (amino acid residues #1-111) was not able to bind the ssRNA (Fig. 17). An interpretation is that the sequence 112-**RMRK**-115 is very important for the ssRNA-binding of VP4.

Although a leucine-zipper motif is found in many nucleic acid binding proteins, it is not required for ssRNA-binding of VP4 (Fig. 14 and Fig. 17). Similar observations were found in the rotavirus VP2 protein (Labbé *et al.*, 1994) and the reovirus sigma 3 protein (Miller and Samuel, 1992).

From the chemical cross-linking experiment (Fig. 19), VP4 was identified as an oligomer in solution. In the case of HDAg, oligomerization is required for RNA replication (Xia and Lai, 1992) but is not required for RNA binding (Lin *et al.*, 1990). When VP4 proteins were cross-linked with glutaraldehyde, only the VP4 monomer and oligomer appeared (Fig. 19). The same results were obtained with other types of cross-linking agents [e.g. ethyl glycol-bis(succinic acid) N-hydroxysuccinimide, dimethyl pimelimidate dihydrochloride] (data not shown). Reducing the concentration of glutaraldehyde to 0.005% or the reaction temperature to 0° C did not inhibit oligomer formation (data not shown). The VP4 oligomer was not able to enter a native gel, but was able to enter the gel in the presence of 0.1% SDS (data not shown). The addition of 400 mM NaCl, 16 mM ß-mercaptoethanol, or 40% glycerol to the protein mixture did not allow VP4 to enter the native gel. This is evidence that the exclusion of VP4 is not the result of nonspecific interactions with other proteins. Although the chemical cross-linking experiments established that VP4 may be an octamer, more data have to be obtained to confirm these results, such as gel filtration chromatography and electron-microscopy using the purified VP4.

The leucine-zipper motif is not required for VP4 oligomerization (Fig. 20). A similar observation was also found in measles virus (Buckland *et al.*, 1992) and Newcastle disease virus (NDV) (Reitter et al., 1995). The leucine-zipper motifs of the measles virus and NDV fusion proteins are not required for their oligomerization but are essential for fusion. Whether the leucine-zipper motif has any function in VP4 is unknown. In the deduced amino acid sequences of BTV VP1 proteins, two leucine-zipper motifs were identified (Huang *et al.*, 1995). Perhaps VP4 interacts with VP1 through the leucine-zipper motifs, and forms a complex to regulate the transcription and replication of BTV.

In the Northwestern assay, the VP4 monomer was able to bind ssRNA (Fig. 16). However, we cannot rule out that during the refolding procedures VP4 formed its oligomer structure. Whether the oligomerization of VP4 is required for its RNA-binding is not clear. The VP4 native structure is required for the RNA binding, since SDS-denatured VP4 lost RNA-binding activity (Fig. 12).

In the study of the *rev* protein of human immunodeficiency virus type 1 (HIV-1), tetramerization is required for RNA binding (Zapp *et al.*, 1991), and both the oligomerization and RNA-binding domains are located at the

arginine-rich regions. Whether VP4 uses the same mechanism for RNA binding and oligomerization is unknown.

Presently, five BTV proteins have been shown to possess ssRNA-binding activity. These include VP1 (Urakawa and Roy, 1989), VP3 (Loudon and Roy, 1992), VP4 (this study), VP6 (Hayama and Li, 1994), and NS2 (Huismans *et al.*, 1987; Thomas *et al.*, 1990; Zhao *et al.*, 1994). Interestingly, none of these BTV proteins bind ssRNA in a sequence-specific manner. This contrasts with the situation in rotavirus, in which some of the RNA-binding proteins (VP1, NSP1, and NSP3) possess specific affinity for rotavirus mRNA, and others (VP2, NSP2, and NSP5) have nonspecific binding activity (Patton, 1995). How BTV specifically encapsidates the 10 mRNAs through these nonspecific RNA-binding proteins still needs to be answered.

REFERENCES

Bandziulis, R.J., Swanson, M.S., and Dreyfuss, G. (1989). RNA-binding proeins as developmental regulators. Genes and Dev. **3**, 431-437.

Borden, E.C., Shope, R.E., and Murphy, F.A. (1971). Physiochemical and morphological relationship of some arthropod-borne viruses to bluetongue virus--a new taxonomic group: Physiochemical and serological studies. J. Gen. Virol. **13**, 261-271.

Bowne, B., Steinberg, J., Laemmli, U.K., and Weintraub, H. (1980). The detection of DNA-binding proteins by blotting. Nucleic Acids Res. **8**, 1-20.

Buckland, R., Malvoisin, E., Beauverger, P., and Wild, F. (1992). Leucine-zipper structure present in the measles virus fusion protein is not required for its tetramerization but is essential for fusion. J. Gen. Virol. **73**, 1703-1707.

Genetics Computer Group (1994). Program manual for the GCG package, version 8., Genetic Computer Group, Madison, Wisconsin.

Hayama, E., and Li, J.K.-K. (1994). Mapping and characterization of antigenic epitopes and the nucleic acid binding domains of the VP6 protein of bluetongue viruses. J. Virol. **68**, 3604-3611.

Hoch, S.O. (1982). DNA-binding domains of fibronectin probed using western blots. Biochem. Biophys. Res. Comm. **106**, 1353-1358.

Huang, I-J., Hayama, E., Hwang, G.-Y., Yang, Y.-Y., and Li, J.K.-K. (1995). Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five U.S. bluetongue viruses. Virology **214**, 280-288.

Huang, I-J., Hayama, E., Jeong, Y.-J., and Li, J.K.-K. (1993). Conservation of the segment 4 gene sequence and of a leucine-zipper motif in VP4 among five US bluetongue viruses. Virology **195**, 772-779.

Huismans, H., and Van Dijk, A.A. (1990). Bluetongue virus structural components. Curr. Top. Microbiol. Immunol. **162**, 21-37.

Huismans, H., Van Dijk, A.A., and Bauskin, A.R. (1987). *In vitro* phosphorylation and purification of a nonstructual protein of bluetongue virus with affinity for single-stranded RNA. J. Virol. **61**, 3589-3595.

Hwang, G.-Y., and Li, J.K.-K. (1993). Identification and localization of a serotypic neutralization determinant on the VP2 protein of bluetongue virus 13. Virology **195**, 859-862.

Johnson, T.M., and Li, J.K.-K. (1991). Heterologous expression and characterization of an active lignin peroxidase from phanerochaete chrysoporium using recombinant baculovirus. Arch. Biochem. Biophys. **291**, 371-378.

Kraft, R., Tardiff, J., Krawter, K.S., and Leinwand, L.A. (1988). Using mini-prep plasmid DNA for sequencing double stranded template with sequenase. BioTechniques **6**, 544-547.

Labbé, M., Baudoux, A., Charpilienne, D., and Cohen, J. (1994). Identification of the nucleic acid binding domain of the rotavirus VP2 protein. J. Gen. Virol. **75**, 3423-3430.

Laemmli, U.K. (1970). Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Landschulz, W.H., Johnson, P.F., and McKnight, M.C. (1988). The leucine-zipper: A hypothetical structure common to a new class of DNA binding proteins. Science **240**, 1759-1764.

Lazinski, D., Grzadzielska, E., and Das, A. (1989). Sequence-specific recognition of RNA hairpins by bacteriophage antiterminators requires a conserved arginine-rich motif. Cell **59**, 207-218.

Le Blois, H., French, T., Mertens, P.P.C., Burroughs, J.N., and Roy, P. (1992). The expressed VP4 protein of bluetongue virus binds GTP and is the candidate guanylyl transferase of virus. Virology **189**, 757-761.

Lee, C.-Z., Lin, J.-H., Chao, M., McKnight, K., and Lai, M.M.C. (1993). RNA-binding activity of hepatitis delta antigen involves two arginine-rich motifs and is required for hepatitis delta virus RNA replication. J. Virol. **67**, 2221-2227.

Li, J.K.-K., and Yang, Y.-Y. (1990). Mapping of two immunodominant antigenic epitopes conserved among the major inner core protein, VP7 of five bluetongue viruses. Virology **178**, 552-559.

Lin, J.H., Chang, M.F., Baker, S.C., Govindarajan, and Lai, M.M.C. (1990). Characterization of hepatitis delta

antigen: Specific binding to hepatitis delta virus RNA. J. Virol. **64**, 4051-4058.

Loudon, P.T., and Roy, P. (1992). Interaction of nucleic acids with core-like and subcore-like particles of bluetongue virus. Virology **191**, 231-236.

McCormack, S.J., Thomas, D.C., and Samuel, C.E. (1992). Mechanism of interferon action: Identification of a RNA-binding domain within the N-terminal region of the human RNA-dependent P1/eIF-2α protein kinase. Virology **188**, 46-56.

Mattaj, I.W. (1989). A binding consensus: RNA protein interactions in splicing, ssRNPs, and sex. Cell *57*, 1-3.

Mattaj, I.W. (1993). RNA recognition: A family matter? Cell **73**, 873-840.

Merrill, B.M., Stone, K.L., Cobianchi, F., Wilson, S.H., and Williams, K.R. (1988). Phenylanines that are conserved among several RNA-binding proteins from part of a nucleic acid binding packet in the A1 heterogeneous nuclear ribonucleoprotein. J. Biol. Chem. **263**, 3307-3313.

Miller, J.E., and Samuel, C.E. (1992). Proteolytic cleavage of the reovirus sigma 3 protein results in enhanced double-stranded RNA-binding activity: Identification of a repeat basic amino acid motif within the C-terminal binding region. J. Virol. **66**, 5347-5356.

Murphy, F.A., Borden, E.C., Shope, R.E., and Harrison, A. (1971). Physiochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus-a new taxonomic group: electron microscopic studies. J. Gen. Virol. **13**, 273-278.

Nagai, K., Oubridge, C., Jessen, T.H., Li, J., and Evans, P.R. (1990). Crystal structure of the RNA-binding domains of the U1 small nuclear ribonucleoprotein A. Nature **348**, 515-520.

Parsell, D.A., Kowal, A.S., and Lindquist, S. (1994). *Saccharomyces cerevisiae* Hsp104 protein: Purification and characterization of ATP-induced structure changes. J. Biol. Chem. **269**, 4480-4487.

Patton, J.T. (1995). Structure and function of the rotavirus RNA-binding proteins. J. Gen. Virol. **76**, 2633-2644.

Perutz, M. (1992). How proteins recognize genes in health and disease. In: Protein structure (M. Sanderson and K. Herb eds.), pp. 77-117. W.H. Freeman and Company, New York.

Reitter, J.N., Sergel, T., and Morrison, T.G. (1995). Mutational analysis of the leucine-zipper motif in the newcastle disease virus fusion protein. J. Virol. **69**, 5995-6004.

Sorger, P.K., and Nelson, H.C.M. (1989). Trimerization of yeast transcriptional activator via a coiled-coil motif. Cell **59**, 807-813.

Tam, T.P. (1988). Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA **85**, 5409-5413.

Thomas, C.P., Booth, T.F., and Roy, P. (1990). Synthesis of bluetongue virus encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: It binds the single-stranded RNA species. J. Gen. Virol. **71**, 2073-2083.

Urakawa, T., and Roy, P. (1989). Expression of the largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: Association of VP1 protein with RNA polymerase activity. Nucleic Acids Res. **17**, 7395-7401.

Van Dijk, A.A., and Huismans, H. (1980). The *in vitro* activation and further characterization of the bluetongue virus-associated transcriptase. Virology **104**, 347-356.

Verwoerd, D.W., Els, H.J., DeVilliers, E.M. and Huismans, H. (1972). Structure of the bluetongue virus capsid. J. Virol. **10**, 783-794.

Waksman, G., Shoelson, S.E., Pant, N., Gowburn, D., and Kuriyan, J. (1993). Binding of a high affinity phosphotyrosyl peptide to the Src Sh2 domain: Crystal

structures of the complexed and peptide-free forms. Cell **72**, 779-790.

Xia, Y.-P., and Lai, M.M.C. (1992). Oligomerization of hepatitis delta antigen is required for both the trans-activating and trans-dominant inhibitory activities of the delta antigen. J. Virol. **66**, 6641-6648.

Yang, Y.-Y., Li, J.-K.-K., Johnson, T.M., Mecham, J.O., and Tam, J.P. (1992). Epitopic mapping of linear and conformation-dependent antigenic determinants on GP5 of five US bluetongue viruses. Virology **188**, 530-536.

Zapp, M.L., Hope, T.J., Parslow, T.G., and Green, M.R. (1991). Oligomerization and RNA-binding domains of the type 1 human immunodeficiency virus rev protein: A dual function for an arginine-rich binding motif. Proc. Natl. Acad. Sci. USA **88**, 7734-7738.

Zhao, Y., Thomas, C., Bremer, C., and Roy, P. (1994). Deletion and mutational analyses of bluetongue virus NS2 protein indicated that the amino but not the carboxy terminus of the protein is critical for RNA-protein interactions. J. Virol. **68**, 2179-2185.

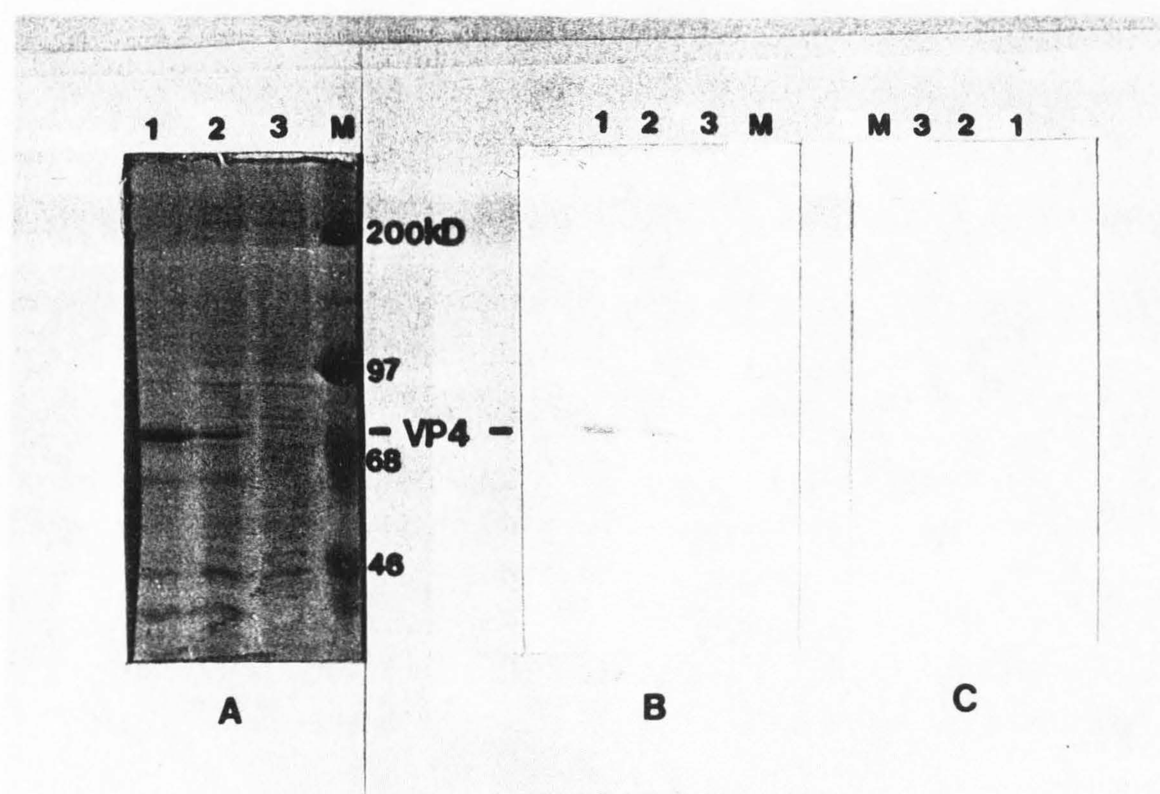


FIG. 6. Expression of VP4 in a baculovirus expression system. (A) Coomassie blue stained SDS-PAGE; Lane 1 and 2: insect cells infected with recombinant baculovirus carrying BTV-13 M1 gene at an M.O.I. of 10 and 5, respectively; Lane 3: mock infected insect cells; Lane M: molecular weight markers. (B) Immunodetection of the same sample as described in (A) with PAb against BTV-13 virion at 1:1,000 dilution. (C) same as (B) except the antibody was pre-immunized rabbit serum as a negative control.

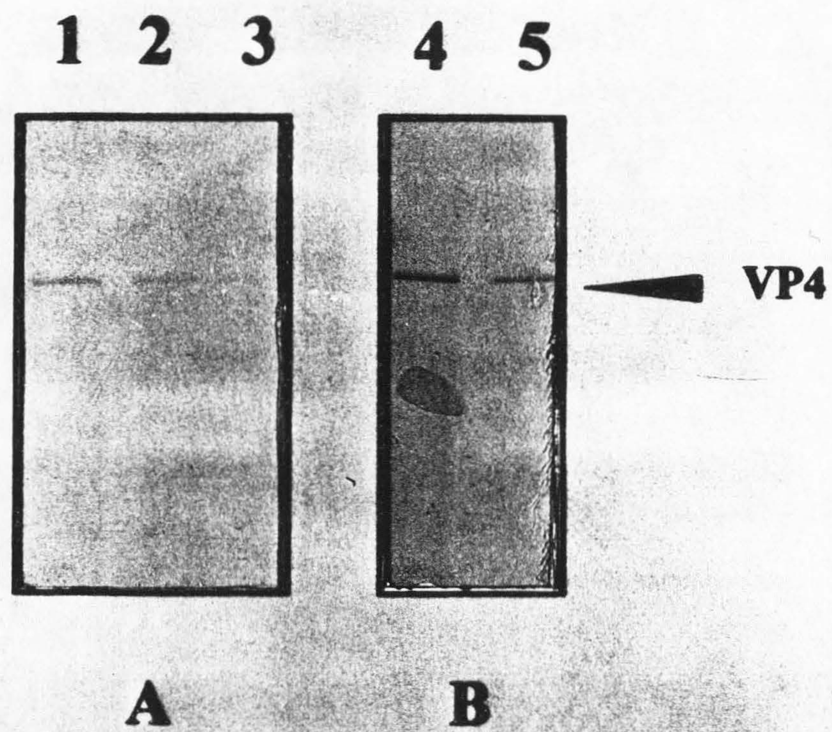


FIG. 7. SDS-PAGE analyses of purified BTV-13 VP4 from the baculovirus expression system. VP4 purified by Sephadex G-75 column was resolved by an SDS-PAGE and stained by (A) Coomassie blue and (B) silver. Lane 1 to 5 are two-fold dilutions of VP4.

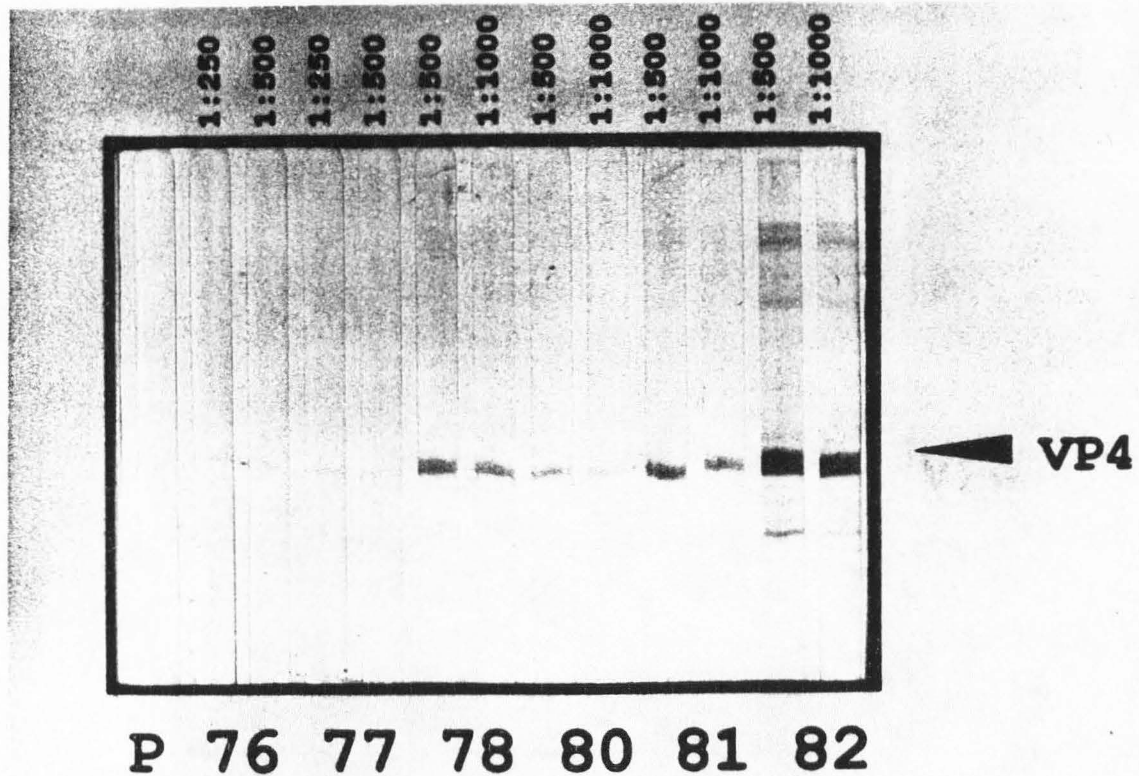


FIG. 8. Western blot immunodetection of VP4 by OAbs. VP4 was transferred to a nitrocellulose membrane after gel electrophoresis through a 7.5% SDS-polyacrylamide gel. The dilutions of OAb #76 and #77 were 1:250 and 1:500; the dilutions of OAbs are showed above the lanes; pre-immuned rabbit serum was used as a negative control (labeled as P).

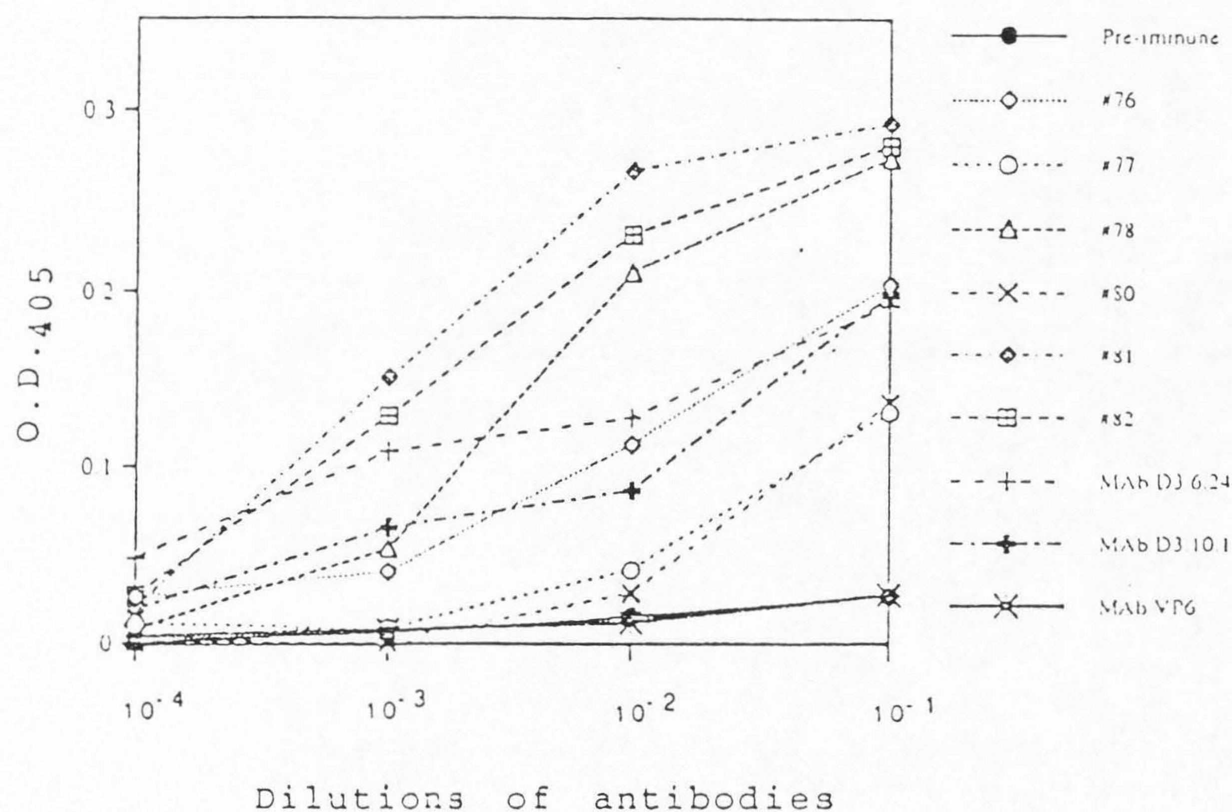


FIG. 9. Binding of anti-VP4 MAbs and OAbs to purified VP4 in ELISA. The degree of binding of these antibodies to VP4 was measured by the binding of alkaline phosphatase-conjugated second antibodies and the optical density at 405 nm. Pre-immuned rabbit serum and anti-VP6 MAb (Hayama and Li, 1994) were used as negative controls. The dilutions of each antibody were 1:10, 1:100, 1:1,000, and 1:10,000. Each point represents the average of three replicates.

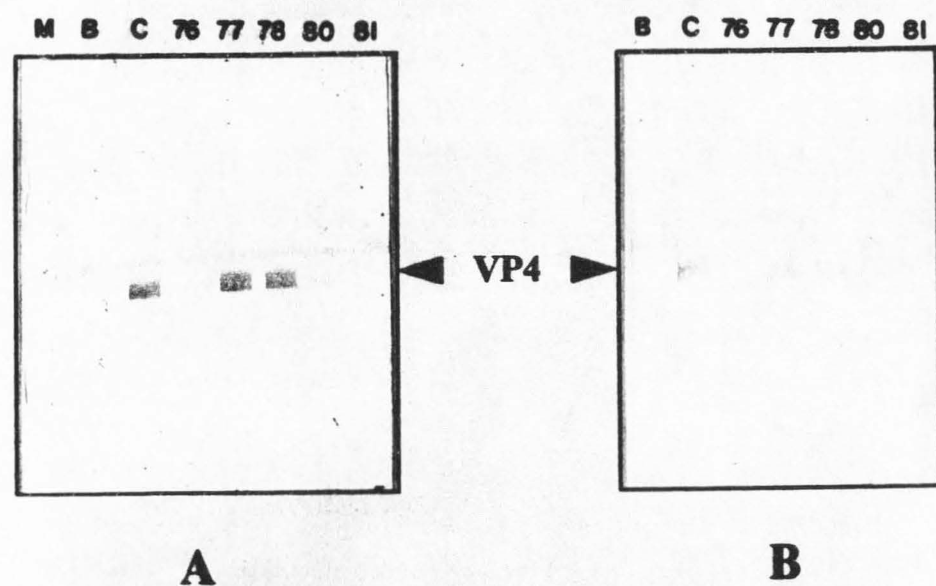


FIG. 10. Competition binding of two anti-VP4 MAbs to VP4 by synthetic peptides. MAb D3.6.24 and D3.10.1 were cross-absorbed with VP4 sequence-specific peptides #76, #77, #78, #80, and #81 before they were used for immunodetection. (A) MAb D3.6.24; (B) MAb D3.10.1. Lane M: pre-stained molecular weight markers (Bio-Rad); Lane B: negative control; Lane C: MAb without cross-absorption. The band across the blots above VP4 is due to the second antibody cross-reaction.

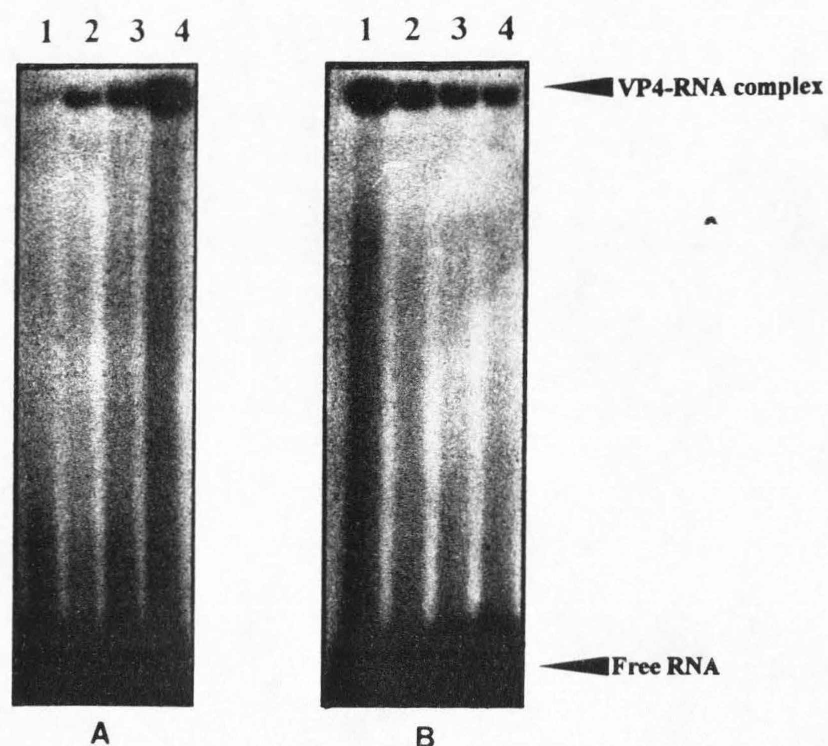


FIG. 11. EMSA of ssRNA-binding activity of VP4. (A) Various amounts of purified BTV-13 VP4 were used to react with [^{32}P]-labeled capped RNA transcripts of BTV M1 gene. Lane 1: labeled transcript without VP4 as control; Lane 2: 20 ng of VP4; Lane 3: 40 ng of VP4; Lane 4: 80 ng of VP4. (B) Competition between capped and non-capped RNA for VP4 binding. Various amounts of unlabeled non-capped BTV-13 M1 transcripts were added to the reaction mixture and the experiment was performed as in (A). Lane 1: no competitor RNA; Lane 2: 100 ng; Lane 3: 200 ng; Lane 4: 400 ng of unlabeled non-capped ssRNA.

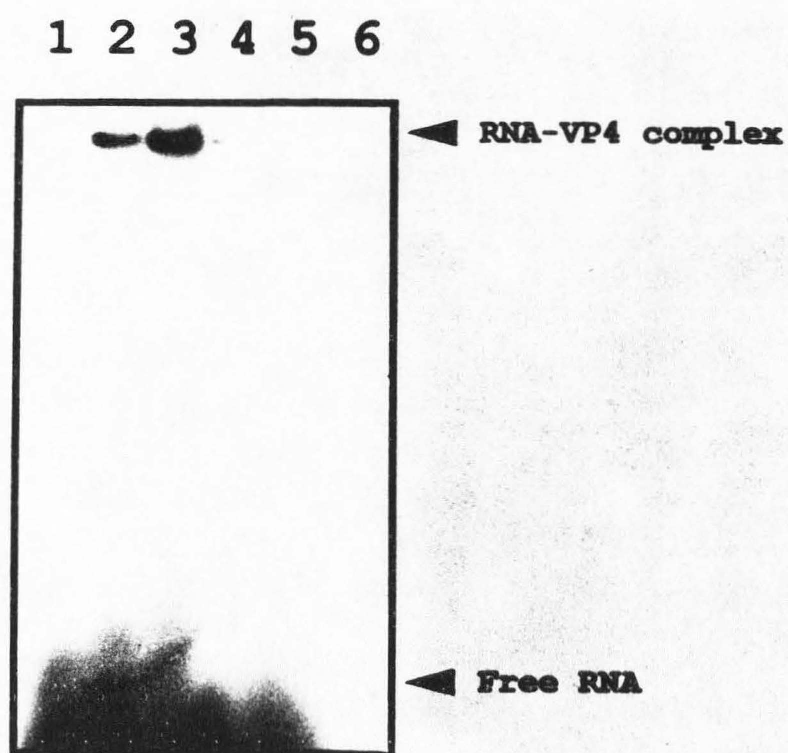


FIG. 12 Competition between tRNA and BTV ssRNA for VP4 binding in EMSA. Lane 1: Labeled [^{32}P]-RNA without VP4 as control; Lane 2: 40 ng of VP4; Lane 3: 80 ng of VP4; Lane 4: 500 µg of yeast tRNA was included in the reaction mixture; Lane 5: same as lane 3 except 1% SDS was added in the reaction mixture; Lane 6: ssRNA was incubated with RNase A.

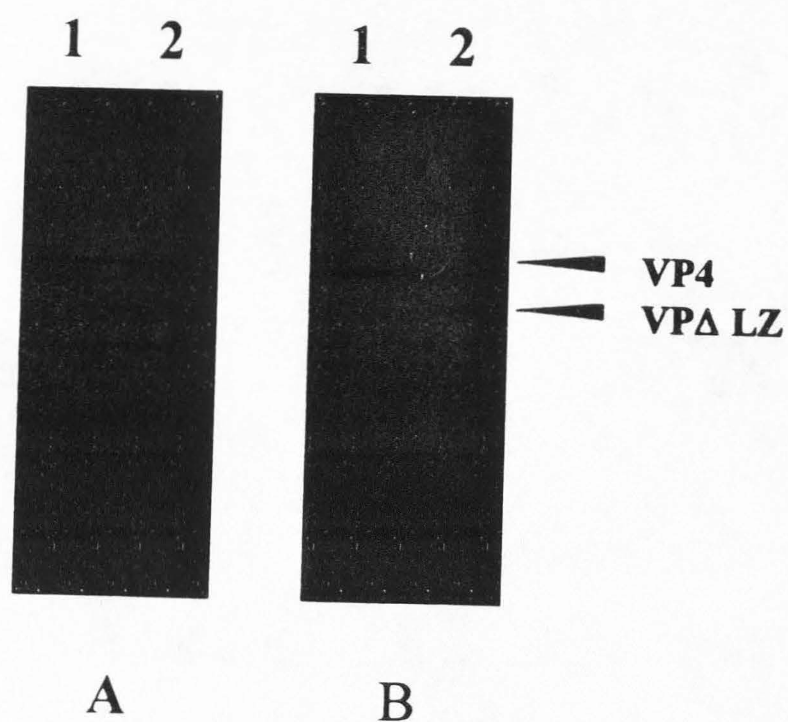


FIG. 13. *In vitro* translation of BTV-13 VP4 and an internal deletion mutant (VP4ΔLZ) with wheat germ extract. (A) Translation of intact VP4 from the capped RNA transcript of the M1 gene (Lane 1) and from non-capped M1 gene transcript (Lane 2). (B) Translations of the intact M1 gene transcript (Lane 1) and internal M1 deletion mutant which lacks the leucine-zipper domain (VP4ΔLZ) (Lane 2).

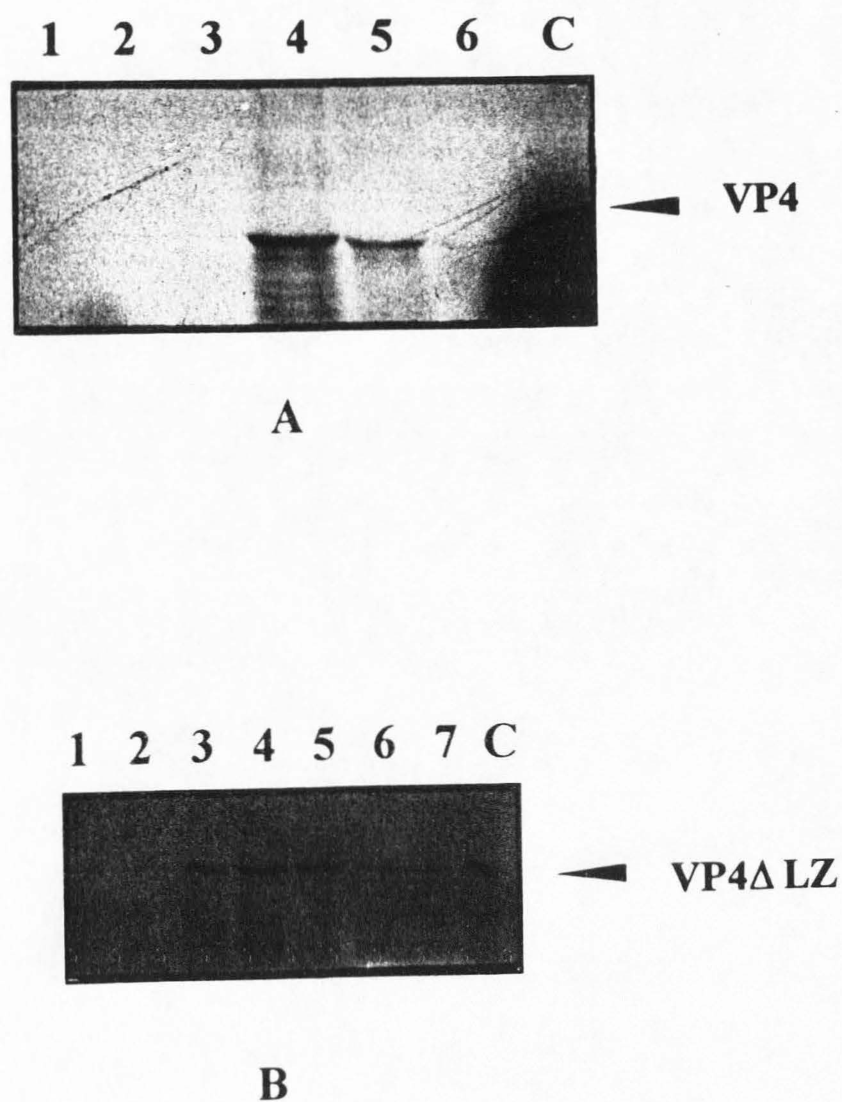


FIG. 14. Profiles of VP4 and VP4ΔLZ eluted from a poly(U)-sepharose column. *In vitro* translated proteins labeled with [^{35}S]-methionine were applied to a poly(U)-sepharose column, eluted with 0.15 M NaCl, and collected. The eluants were precipitated with TCA and resolved in SDS-PAGE and autoradiographed. The numbers indicate fraction numbers. (A) Intact VP4; (B) Truncated VP4 with leucine-zipper region deleted (VP4ΔLZ).

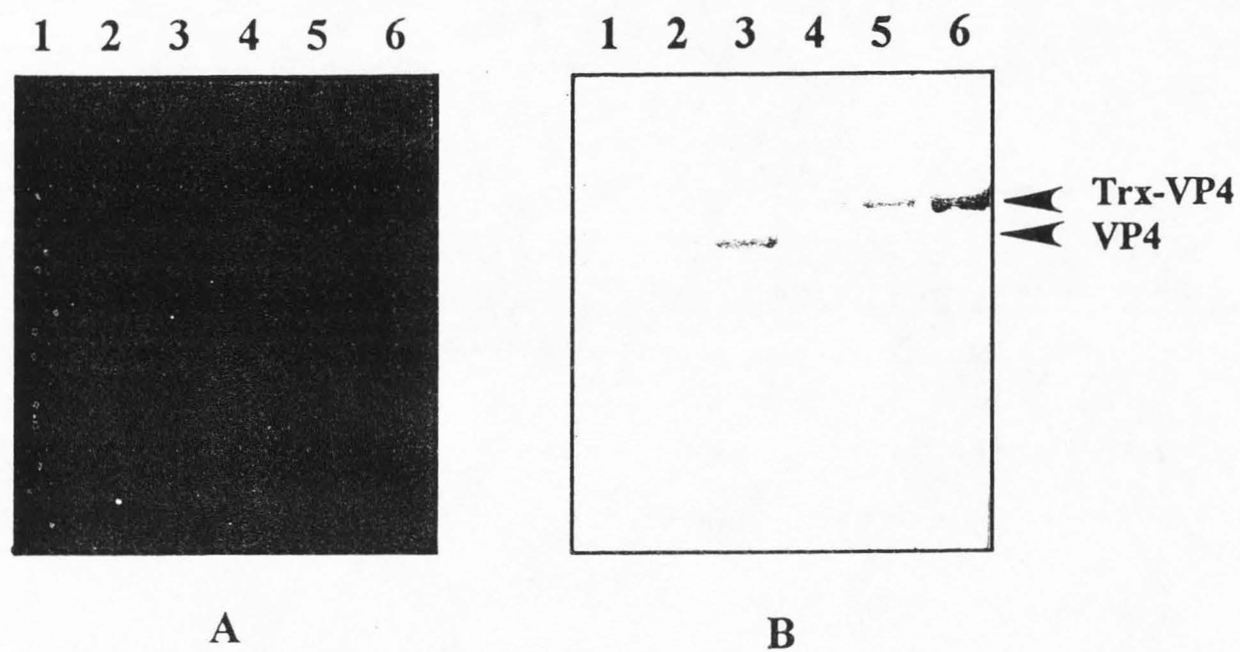


FIG. 15. Northwestern assay of VP4 expressed in insect cells and *E. coli*. VP4 and Trx-VP4 are indicated by arrow heads. (A) Expressed VP4 probed with [^{32}P]-ssRNA. (B) Immunodetection to the same membrane with OAb #82. Lane 1-3 were VP4 expressed in SF-9 cells with about 0.5, 1, and 2 µg of proteins, respectively. Lane 4-6 were Trx-VP4 fusion proteins expressed in *E. coli* with about 0.5, 1, and 2 µg, respectively.

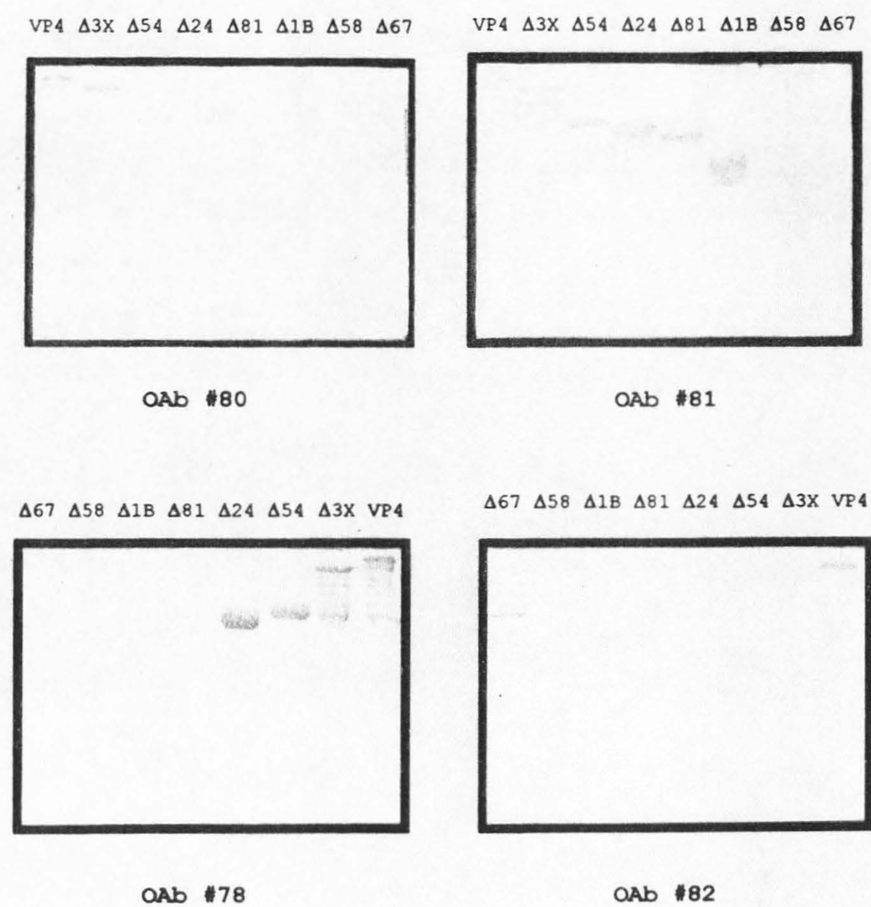


FIG. 16. Immunodetection of Trx-VP4 and the truncated VP4 proteins. OAbs # 78, 80, 81, and 82 were used for the detection at a dilution of 1:1000.

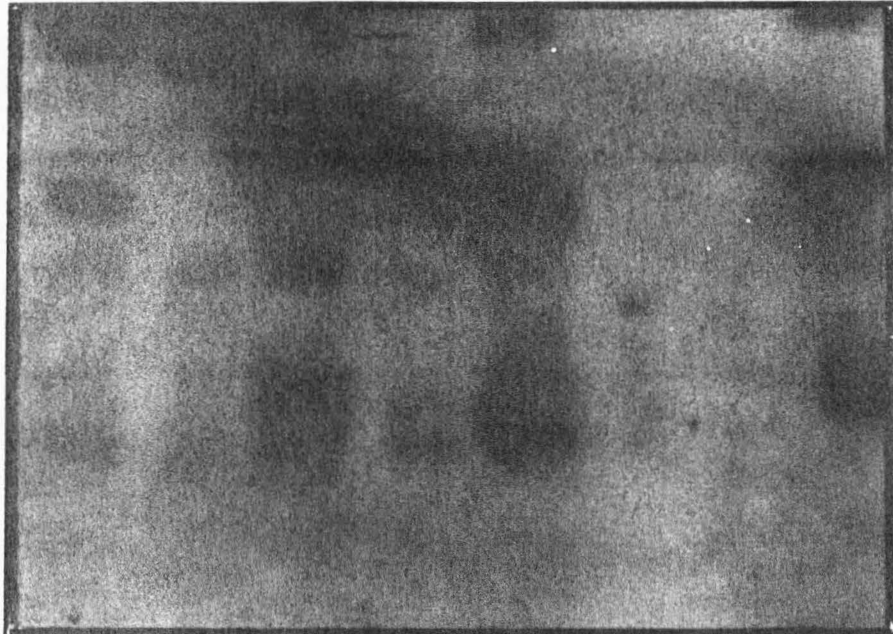
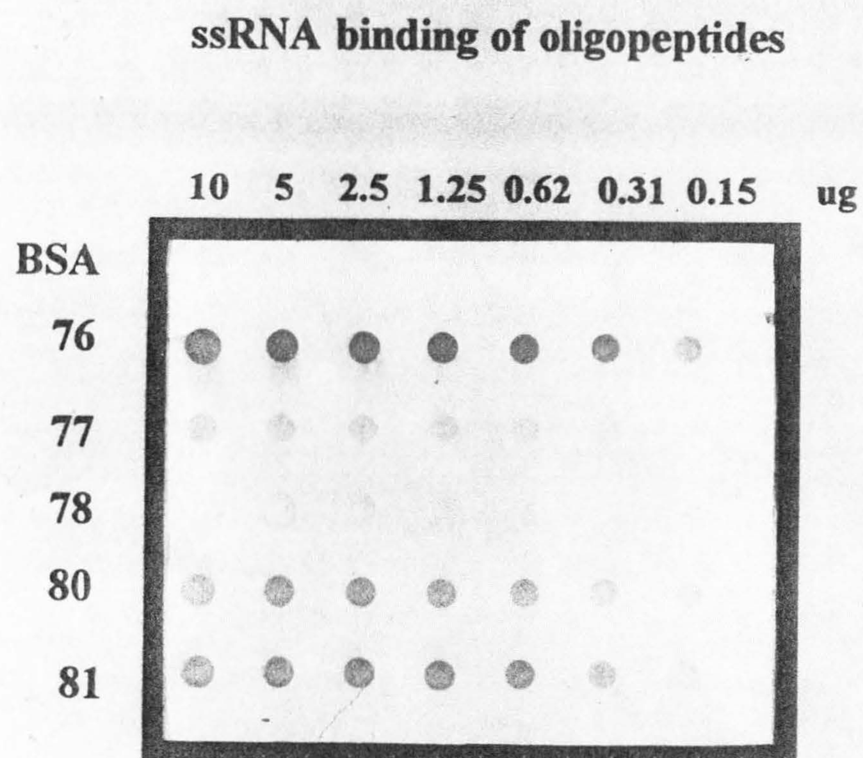


FIG. 17. Mapping the ssRNA-binding domains of VP4 with Northwestern assay. [^{32}P]-ssRNA was used to probe immobilized VP4 and the truncated VP4 proteins on a nitrocellulose membrane.



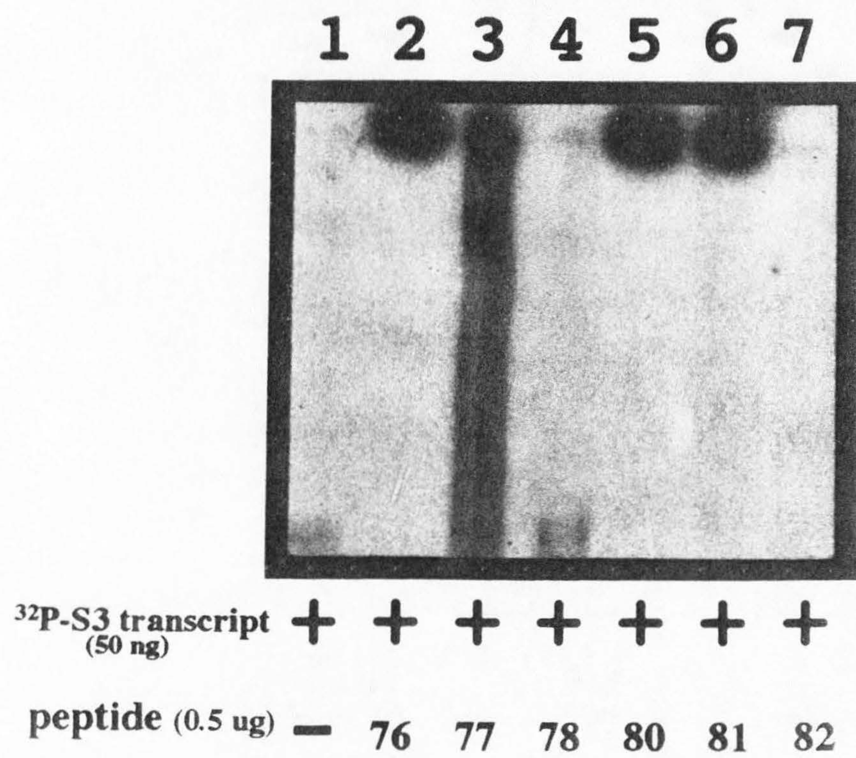


FIG. 18. ssRNA-binding activity of VP4 sequence-specific peptides. (A) Northwestern assay. Biotinylated ssRNA was used to probe the immobilized peptides on nitrocellulose membrane. (B) EMSA.

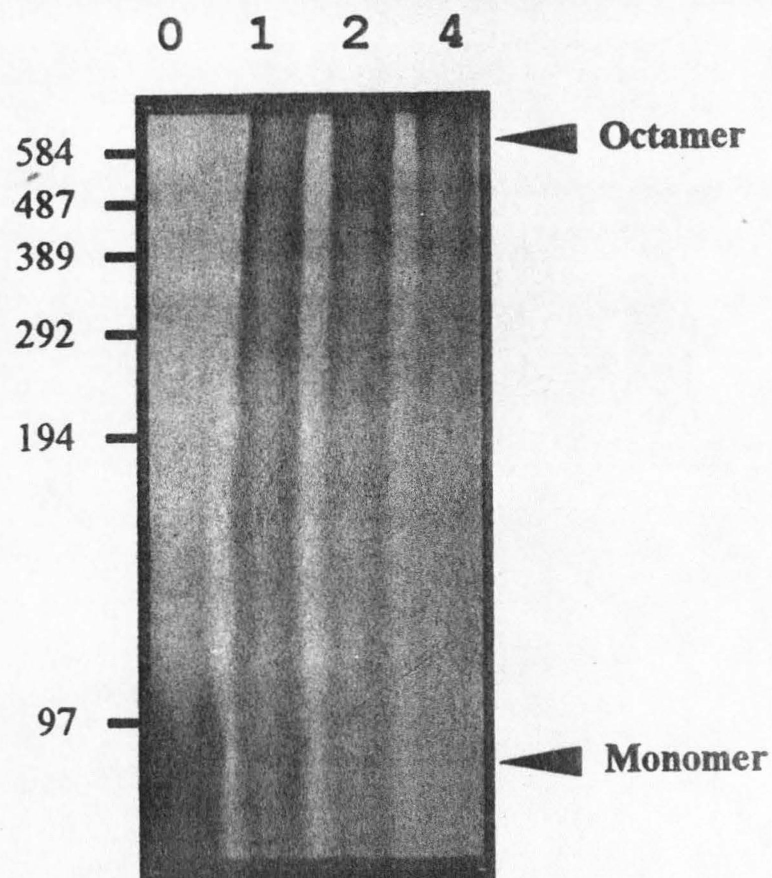


FIG. 19. Chemical cross-linking of *in vitro* translated VP4. Translated VP4 was incubated with 0.01% glutaraldehyde for 1, 2, and 4 min at room temperature. Cross-linked products were analyzed by 3.5% SDS-PAGE.

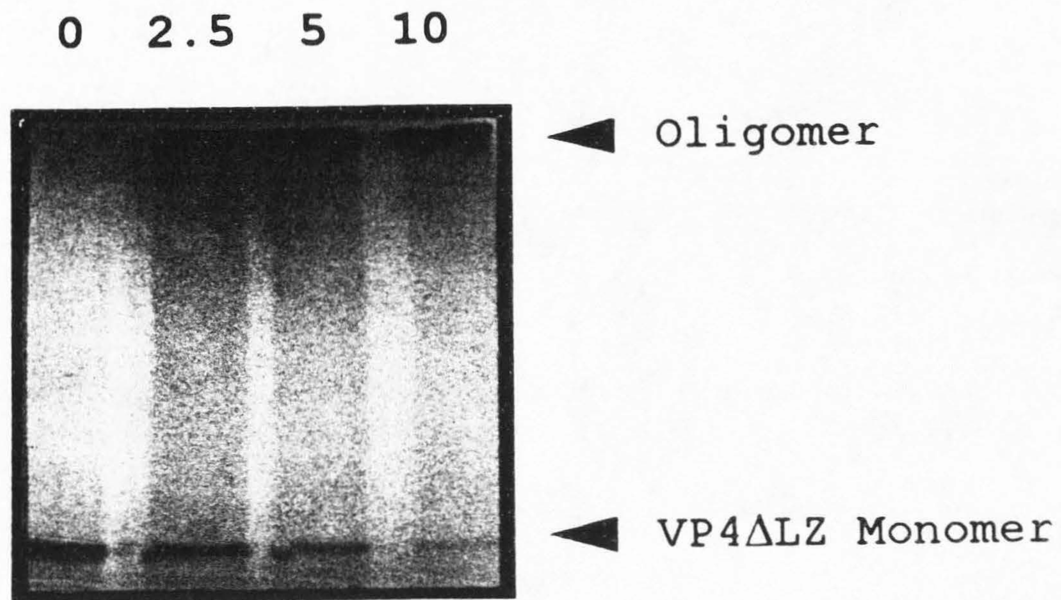


FIG. 20. Chemical cross-linking of VP4 with deleted leucine-zipper motif (VP4ΔLZ). *In vitro* translated truncated VP4 protein lacks the leucine-zipper motif (VP4ΔLZ) was incubated with 2.5, 5, and 10 mM of DMS at room temperature for 25 min and analyzed on 7.5% SDS-PAGE.

CHAPTER IV

CONCLUSION AND DISCUSSION

Bluetongue virus (BTV) is the agent that causes bluetongue disease in ruminants. The mechanism of BTV replication is still largely unknown. The transcription of BTV ssRNA and the replication of BTV dsRNA were assumed to be controlled by three inner core proteins: VP1, VP4, and VP6. The functions of these inner core proteins are still not clear. VP1 is the potential RNA-dependent RNA polymerase (Urakawa and Roy, 1989). VP6 is a DNA- and RNA-binding protein (Hayama and Li, 1994). VP4 is another minor inner protein of BTV, and its function is unknown. To understand the replication of BTV, the function of each viral protein must be known. In this study, the gene (M1) coding for VP4 was cloned and sequenced. VP4 was expressed and purified in order to study its functions.

Phylogenetic study of BTV using M1 genes

The length of five M1 genes determined in this study is 1981 nucleotides, 30 nucleotides shorter than that of the BTV serotype-10 reported by Yu *et al.* (1987). The 5'- and 3'-noncoding regions of all five M1 genes are identical among all serotypes. The M1 gene possesses a single, long ORF that

encodes 644 amino acid residues. A potential leucine-zipper motif was identified near the carboxyl terminus of the deduced VP4 amino acid sequence. There is a high degree of sequence conservation among the five US BTV VP4 proteins with fewer than 4% mismatches in their amino acid sequences (Table 2). The phylogenetic analysis using the sequences of these five cognate M1 genes yielded results consistent with our previous phylogenetic studies of cognate genome segments 1, 3, 5, 6, 8, 9, and 10. Serotypes-10, -11, -13, and -17 are closely related, and serotype-2 is the most distantly related among the five US BTVs (Fig. 4).

Characterization of VP4

Recombinant VP4 was expressed and found to be highly insoluble in both eukaryotic (insect cells and yeast) and prokaryotic (GST-, Trx-, and 6xH-fusion proteins) systems. When detergents and reducing agents were combined, the heterologously expressed VP4 was able to be solubilized and purified to near homogeneity (Fig. 7). The recombinant VP4 possessed a ssRNA-binding activity in both the EMSA (Fig. 11) and the Northwestern assay (Fig. 15). VP4 protein has binding activity to both capped and non-capped ssRNAs. Its RNA-binding activity was not specific to BTV ssRNA as it was demonstrated that *in vitro* translated VP4 could bind to

poly(U)-sepharose (Fig. 14), and its BTV ssRNA-binding activity could be competed out by yeast tRNA (Fig. 12). Although a leucine-zipper motif is found in many proteins (summarized in Table 5) including nucleic acid binding proteins, the leucine-zipper of VP4 is not required for the RNA binding (Fig. 14 and 17).

An RNA-binding domain was mapped between amino acid residues #112-158. An N-terminal truncated VP4 was constructed (mutant Δ67) to investigate whether residues #112-158 are the only ssRNA-binding domain. The mutant Δ67, which lacks amino acid residues #112 to 158, retained ssRNA-binding activity (Fig. 17). This suggests that more than one ssRNA-binding domain exists in VP4.

To identity other potential ssRNA-binding domains of VP4, sequence-specific synthetic peptides corresponding to VP4 in the arginine- and lysine-rich regions were used in EMSA and Northwestern assays (Fig. 18). These regions are located at amino acid residues #103-115 (ENEIGRRRIRMRK), #376-385 (RDRGTFLKKR), #583-592 (KEQKLRDLKR), and #617-626 (RALREYKRK). These sequences are rich in basic amino acid residues (R and K), which are found in many of the RNA-binding proteins (Merrill *et al.*, 1988; Lazinski *et al.*, 1989; Nagai *et al.*, 1990). These regions are also surface accessible on VP4 (Fig. 9 and 21) and thus are more

available for binding to RNA. The RNA-binding domains are summarized in Fig. 23.

VP4 was suggested as an oligomer based on chemical cross-linking experiments (Fig. 19), but additional data have to be collected to confirm this conclusion. The leucine-zipper motif is not required for the oligomerization (Fig. 20), although it is responsible for oligomerization in some other proteins (Landschulz *et al.*, 1988; Sorger and Nelson, 1989). Whether the oligomerization of VP4 is required for its RNA-binding activity is not clear. The nondenatured form of VP4 is required for the RNA-binding; in the presence of SDS, VP4 was not able to bind ssRNA in EMSA (Fig. 12).

Mapping the antigenic epitopes of VP4

Six antigenic epitopes were mapped at amino acid residues #617-626 (RALREYKRKM), #583-592 (KEQKLRDLKR), #167-178 (GARFDDEPTDEK), #376-385 (RDRGTFLKKR), #103-115 (ENEIGRRRIRMRK), and #523-551 (RVESSVLRVRNPTLHETADELK RMGLDL) (Fig. 8). These regions are also surface accessible as shown in ELISA (Fig. 9). Peptide competition assays for blocking two monoclonal antibodies (D3.6.24 and D3.10.1) also confirmed that #617-626, #376-385, and #103-

115 are surface accessible (Fig. 9) and that the two antigenic determents are conformational.

Potential function of VP4

Although VP4 is the candidate for being the BTV guanylyltransferase, its sequence is not similar to any of the known guanylyltransferases and capping enzymes. Using the FASTA program of GCG to search for homology between VP4 and other known proteins, I found that amino acid residues between #7 and #41 have 47.6% identity in a 21-amino acid overlap to the RNA-directed RNA polymerase of human respiratory syncytial virus, which is a ssRNA virus. This region also has 33.3% identity in a 21-amino acid overlap to the RNA-directed RNA polymerase of kunjin virus, which is also a ssRNA virus (Fig. 22A). Another region between amino acid residues #533-587 was also found to have 27% identity in a 37-amino acid overlap to the ß-subunit of the RNA polymerase of vesicular stomatitis virus, which is a ssRNA virus. The same region also has 37.5% of identity in a 16-amino acid overlap to the RNA-directed RNA polymerase (ORF1A) of coronavirus (Fig. 22B). One of the common characteristics of these RNA polymerases is that they are multifunctional proteins. They may be involved in RNA synthesis, capping, and methylation. Perhaps VP4 interacts with VP1, the BTV RNA

polymerase, to form the transcription and replication complex through leucine-zipper motifs predicted from both proteins.

Future research of VP4

This is the first report in which the BTV VP4 protein has been purified to near homogenity and identified as a ssRNA-binding protein, although the structure and functional domains were mapped using recombinant VP4 proteins and sequence-specific peptides. To confirm the RNA-binding domains of VP4, crystallization is required. The technique developed for purifying VP4 can be used to prepare large amounts of VP4 for crystallization. The same technique may also be applied to purify other BTV proteins that are expressed as insoluble forms.

REFERENCES

Hayama, E., and Li, J.K.-K. (1994). Mapping and characterization of antigenic epitopes and the nucleic acid-binding domains of the VP6 protein of bluetongue viruses. J. Virol. **68**, 3604-3611.

Landschulz, W.H., Johnson, P.F., and McKnight, M.C. (1988). The leucine-zipper: A hypothetical structure common to a new class of DNA binding proteins. Science **240**, 1759-1764.

Lazinski, D., Grzadzielska, E., and Das, A. (1989). Sequence-specific recognition of RNA hairpins by bacteriophage antiterminators requires a conserved arginine-rich motif. Cell **59**, 207-218.

Merrill, B.M., Stone, K.L., Cobianchi, F., Wilson, S.H., and Williams, K.R. (1988). Phenylanines that are

conserved among several RNA-binding proteins from part of a nucleic acid binding packet in the A1 heterogeneous nuclear ribonucleoprotein. J. Biol. Chem. **263**, 3307-3313.

Nagai, K., Oubridge, C., Jessen, T.H., Li, J., and Evans, P.R. (1990). Crystal structure of the RNA-binding domains of the U1 small nuclear ribonucleoprotein A. Nature **348**, 515-520.

Park, W.Y., and Seo, J.S. (1995). Leucine zipper-like domain regulates the autophosphorylation and the transforming activity of p130-gag-fps. Biochem. Biophys. Res. Comm. **211**, 447-453.

Qian, Z., Brunovkis, P., Rauscher, F., Lee, L., and Kung, H.J. (1995). Transactivation activity of Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected transformed T cells. J. Virol. **69**, 4037-4044.

Sorger, P.K., and Nelson, H.C.M. (1989). Trimerization of yeast transcriptional activator via a coiled-coil motif. Cell **59**, 807-813.

Urakawa, T., and Roy, P. (1989). Expression of the largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity. Nucleic Acids Res. **17**, 7395-7401.

Yin, M.J., Paulssen, E.J., Seeler, J.S., and Gaynor, R.B. (1995). Protein domains involved in both *in vivo* and *in vitro* interactions between human T-cell leukemia virus type 1 tax and CREB. J. Virol. **69**, 3420-3432.

Yu, Y., Fukusho, A. and Roy, P. (1987) Nucleotide sequence of the VP4 core protein gene (M4 RNA) of US bluetongue virus serotype 10. Nucleic Acids Res. **15**, 7206.

TABLE 5

Summary of the Functions of Leucine-Zipper

Motif in Some of the Known Proteins

Protein	Function	Reference
Yeast, heat shock transcription factor	Trimerization	Sorger and Nelson, 1989
Yeast, GCN4	Dimerization DNA binding	Pu and Struhl, 1991
Reovirus, sigma 1 protein	Trimerization	Leone *et al.*, 1991
Hepatitis delta antigen	Dimerization RNA replication	Xia and Lai, 1992
Measles virus, fusion protein	Fusion	Buckland *et al.*, 1992
Newcastle disease virus, fusion protein	Fusion	Reitter *et al.*, 1995
Fujinami sarcoma virus, p130-gag-fps	Trans-activation of tyrosine kinase	Park and Seo, 1995
Marek's disease virus, Meq protein	Dimerization	Qian *et al.*, 1995
Human T-cell leukemia virus type-1, CREB1	Protein-Protein interaction	Yin *et al.*, 1995

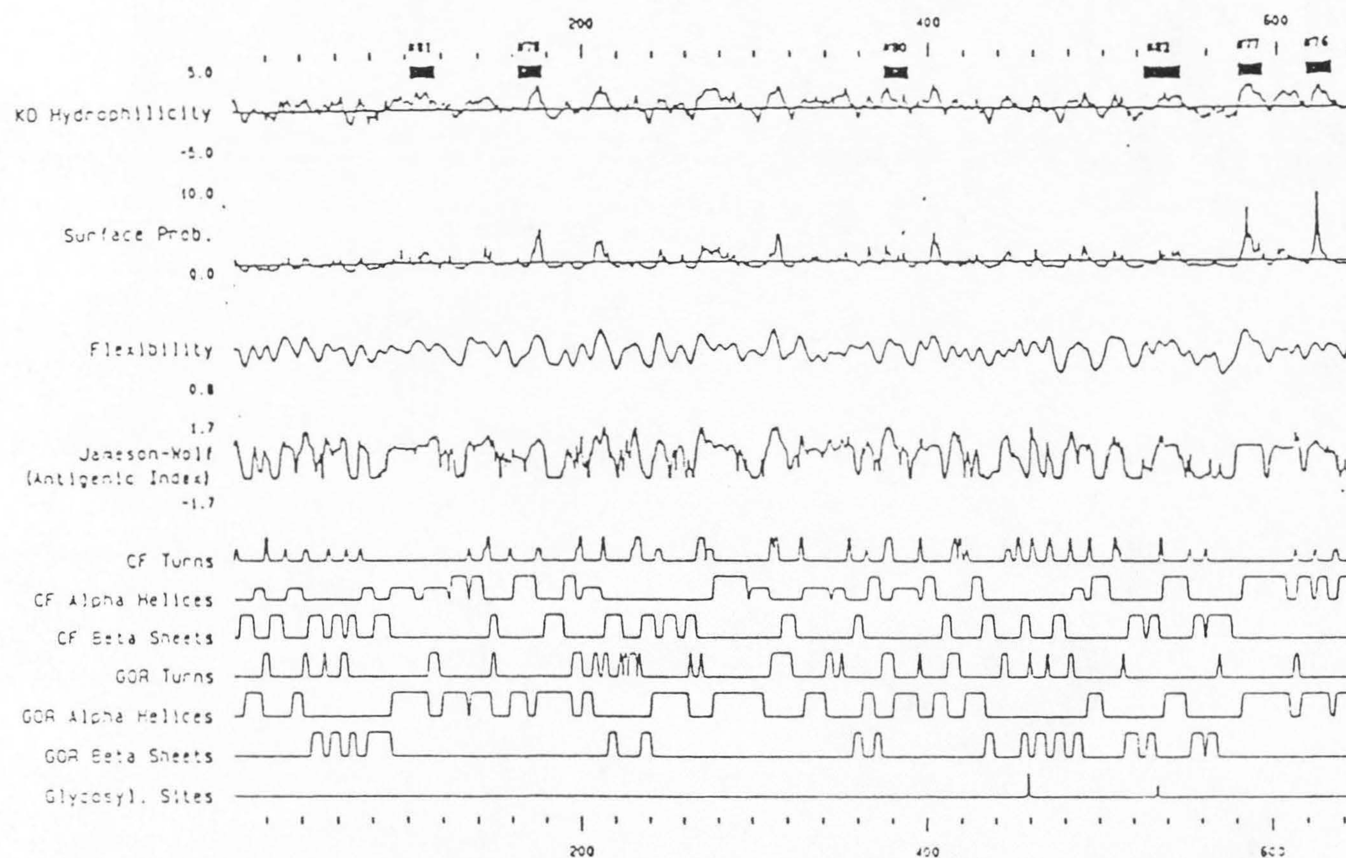


FIG. 21. Protein structure prediction and epitope locations of VP4. The secondary structure of VP4 was predicted using the PlotStructure program of GCG software. The locations of six epitopes were marked as black blocks.

```
BTV-13 VP4      7-VLYVTNELSH.IVKNG.FLPIWKLTGDESLNDLWLEN
Rrpl_Hrsva    389-SDNIINGR..WIILLSKFLKLIKLAGDNNLNNLS.EL
Polg_Kunjm    970-NLAIHSDLSYWIESR..FNDTWKLERAVLGEVKSCTW
```

A

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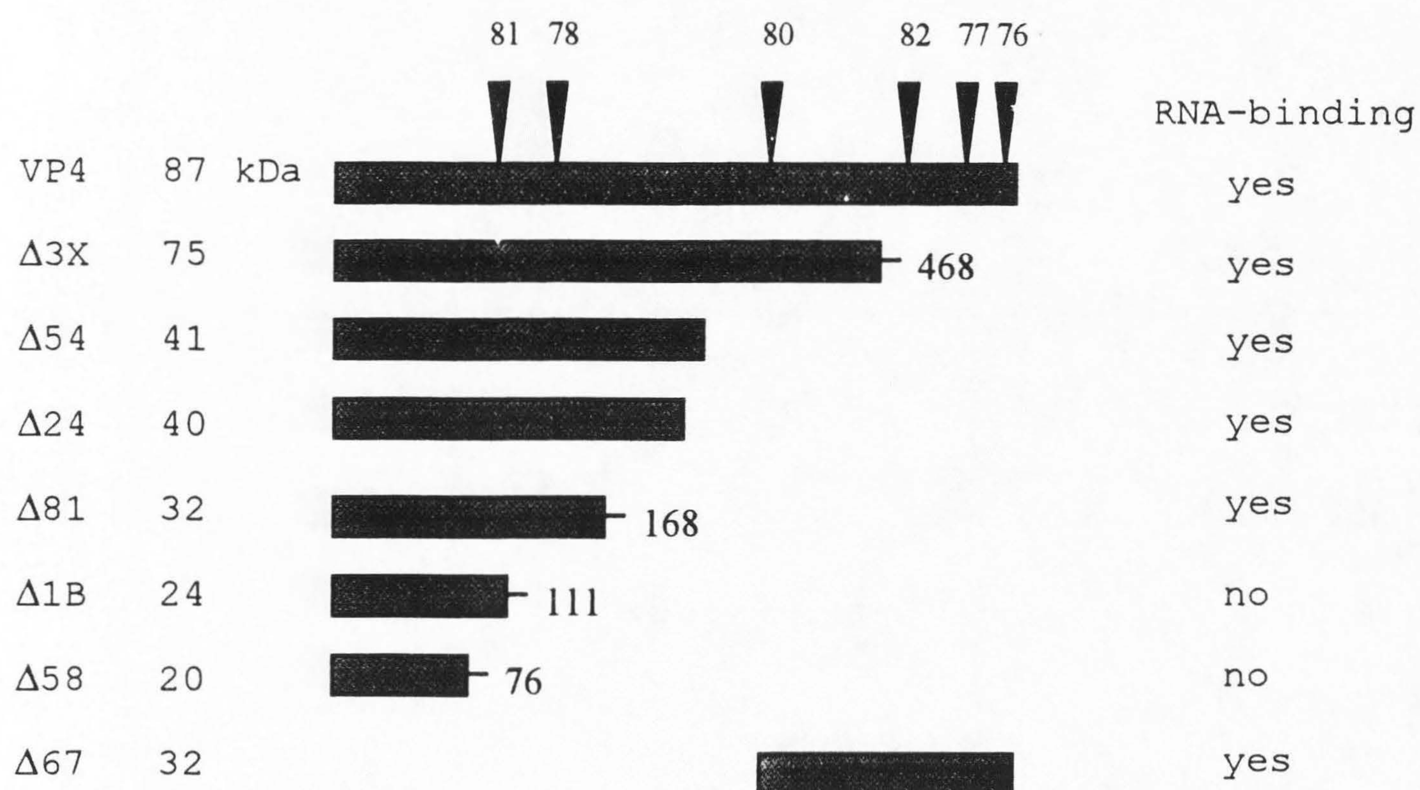
BTV13 VP4    533-RNPTLHETADEL.KRMGLDLSGHLYVTLMSGAYV.TDLFWWFNIILD.W.SAQNKEQKL
Rrpl_Vsvsj   874-KYAMLYLDPS.IGGVSGMSLSRFLIRAFPDPVTESLS.FWRF.IHVHAR.SEHLKEMSA
Rrpa_Cmvjh  3695-LGVTLCALCFVIFA.MLLIKHKHLYLT.MYIMPVLCTLFYTNYLVVGYKQSFRGLAYAW
```

B

FIG. 22. Sequence alignment of VP4 to other proteins using FASTA program of GCG software. (A) The amino acid residues between #7 to 41. Rrpl_Hrsva: RNA polymerase of human respiratory syncytial virus; Polg_Kunjm: RNA polymerase of kunjin virus. (B) The amino acid sequence between #533 to 587. Rrpl_Vsvsj: RNA polymerase of vesicular stomatitis virus; Rrpa_Cmvjh: RNA polymerase of coronavirus. The identical amino acids are bolded and underlined, and numbers at left show residue numbers.



Synthetic peptides		Locations	RNA-binding
#76	RALREYKRKM	#617-626	yes
#77	KEQKLRDLKR	#583-592	yes
#78	GARFDDEPTDEK	#167-178	no
#80	RDRGTFLKKR	#376-385	yes
#81	ENEIGRRRIRMRK	#103-115	yes

FIG. 23. Summary of the truncated VP4 proteins and the VP4 RNA-binding domains. The locations of the peptides are indicated as arrow heads and are also summarized at the bottom of the figure.

APPENDICES

APPENDIX A.

RELATED RESEARCH PUBLICATIONS

APPENDIX A.1

SEQUENCE ANALYSES AND ANTIGENIC EPITOPE MAPPING OF THE PUTATIVE RNA-DIRECTED RNA POLYMERASE OF FIVE U.S. BLUETONGUE VIRUSES[1]

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(Published in 1995: Virology 214, 280-288)

ABSTRACT

We determined the complete nucleotide sequences of the cognate L1 double-stranded RNA segment of bluetongue virus (BTV) serotypes 2, 11, 13, and 17, which encode the putative RNA-directed RNA polymerase VP1. Each L1 gene contained 3944 nucleotides and was 10 bases shorter than the previously reported L1 gene of BTV 10. A single open frame which could encode the reported VP1 protein, 1302

amino acids in size, began with an initiation codon at nucleotides 12-14 and a termination codon at nucleotides 3918-3920. Analyses of the nucleotides of L1 gene and the deduced amino acid sequences of VP1 proteins of the five U.S. BTV serotypes indicated that the most recently isolated BTV-2 serotype from Florida was more distantly related than BTV-10, -11, -13, and -17, which were isolated primary in the western U.S.A. The results are consistent with our hypothesis that BTVs-10, -11, -13, and -17 are derived form a single and common gene pool, and that BTV-2 belongs to a second, distinct gene pool. These genetic distinctions also reflected well with the known geographic distribution of the five U.S. BTV serotypes in North America. This putative RNA-directed RNA polymerase (149 kDa) was a basic protein, and the deduced amino acid sequences of the VP1 proteins contained seven highly conserved hydrophobic domains and many other sequence motifs which were also found in other known RNA polymerases. Four immunodominant but linear antigenic epitopes conserved among the VP1 of five U.S. BTVs were also been identified and mapped using monospecific oligoclonal antibodies.

APPENDIX A.2

CONSERVATION OF THE SEGMENT 4 GENE SEQUENCE AND OF A LEUCINE ZIPPER MOTIF IN VP4 AMONG FIVE US BLUETONGUE VIRUSES[1]

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(Published in 1993: Virology 195, 772-779)

P.S. The results of this paper represent part of this dissertation.

ABSTRACT

Full-length cDNA copies of the segment 4 (M1) genes of US Bluetongue viruses serotype-2, -10, -11, -13, and -17 were selectively amplified using genomic double-stranded RNA segments from purified BTV virions as templates and a modified polymerase chain reaction (Clamp-R). They were then cloned into pUC19 plasmids and both strands of several clones were sequenced. The length of all five segment 4 genes is 1981 nucleotides, which is 30 nucleotides shorter than that

of the BTV serotype-10 reported by Yu *et al*. The 5'- and 3'-noncoding regions of all five segment 4 genes are identical among all serotypes. The plus sense strand of the BTV segment 4 gene, which encodes the VP4 protein, possesses a single long open reading frame with an initiation codon (ATG) at nucleotides #9-11 and a stop codon (TAA) at nucleotides #1941-1943. This open reading frame encodes for a protein of 644 amino acid residues with a predicted molecular weight of about 75 kDa and a pI of +7 to +7.9. A potential leucine zipper motif was detected near the carboxyl terminus of the deduced VP4 amino acid sequence. The phylogenetic analysis using the sequences of these five cognate segment 4 genes is consistent with the results of our previous phylogenetic studies of cognate genome segments 5, 6, 8, 9, and 10. Serotype-10, -11, -13, and -17 are closely related and serotype-2 is the most distantly related among the five US BTV serotypes.

APPENDIX A.3

EVOLUTIONARY ANALYSES OF FIVE US BLUETONGUE VIRUSES USING THE COGNATE S2 GENES[1]

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(Published in 1992: Virus Research 25, 241-249)

ABSTRACT

Full-length cDNA copies of S2 genes (segment 8), coding for nonstructural protein 2 (NS2) of bluetongue virus serotypes 2, 11, 13, and 17 were selectively synthesized by a modified polymerase chain reaction (Clamp-R) and cloned into the Pst I site of the pUC19 plasmid. Each of these S2 cognate genes was 1125 nucleotides in length with an initiation and a termination codon at nucleotides #20-22 and #1082-1084, respectively, resulting in a long open reading frame capable of coding a protein of

354 amino acids. The deduced amino acid sequence of NS2 protein had a high concentration of lysine and contained a relatively low number of tryptophan and histidine residues. There was a highly conserved hydrophilic region at the carboxyl termini of predicted NS2 proteins in all five BTV serotypes, even though the amino acid sequence in this region in BTV-2 was more variable than in the other four serotypes. There was significant sequence homology of the cognate S2 genes at both the nucleotide and the amino acid levels. Phylogenetic analyses using the S2 gene sequences indicated that BTV-10, -11, -13, and -17 were more closely related and BTV-2 was the most distantly related serotype among the five US bluetongue viruses.

APPENDIX B.

EXPRESSION AND PURIFICATION OF BTV-13 VP4 IN *E.COLI*

EXPRESSION AND PURIFICATION OF BTV-13 VP4 IN *E.COLI*

MATERIALS, METHODS, AND RESULTS

Expression of VP4 as GST fusion protein

Full-length cDNA of the BTV-13 M1 gene was amplified as described in CHAPTER II with a pair of primers anchored with a Sma I site (5'-GACGTCGACCCGGGATGCCTGAGCCA-3' and 5'-GACGTCGACCCGGGTAAGTTGTACAT-3'). The amplified cDNA was cloned into the pGEX2T expression vector (Pharmacia) at the Sma I site using the procedures described in CHAPTER II. The orientation of the insert was checked by BamH I enzyme digestion. The plasmid containing BTV-13 M1 cDNA (pGEX2TBTV13M1) was then transformed into *E. coli* TOPP2 strain (Stratagene) as described in CHAPTER II for expression. The expressed VP4 was a GST-VP4 fusion protein.

One ml of overnight culture grown in LB containing 50 µg/ml of ampicillin at 34° C was diluted 8-fold with LB and allowed to grow for 1.5 hr. Isopropyl-ß-D-thiogalacto-pyranoside (IPTG) was added to the culture at a final concentration of 0.05 mM as an inducer to initiate the protein expression. After 2.5 hr of induction at 34° C,

the cells were harvested by centrifugation at 4,000 rpm for 10 min and resuspended in 1 ml of ice-cold MTPBS (150 mM NaCl, 16 mM Na_2PO_4, 4 mM NaH_2PO_4, pH 7.3) plus 50 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), and 1% TX-100. The cells were then lysed by sonication (VirSonic, 80% power output) three times (30 sec each) at 4° C and centrifuged at 4,000 rpm for 15 min to separate the soluble and insoluble fractions. The expressed GST-VP4 was found mainly in the insoluble fraction (data not shown). By lowering the temperature to 15° C for growing and expression, the production of soluble GST-VP4 protein increased. However, the overall amount of protein production was reduced tremendously (data not shown).

The insoluble pellets were resuspended in 1 ml of 25 mM triethanolamine, pH 8, 1mM EDTA, and 1.5% N-lauroyl sarcosine and incubated at 4° C for 25 min. TX-100 was then added to the protein solution to a final concentration of 1%. The solution containing GST-VP4 was then incubated with 20 µl of glutathione-conjugated agarose beads (G-beads, Sigma), pre-equilibrated with the same buffer at 4° C, for 1 hr to extract the GST-VP4 fusion protein. The GST-VP4 bound to the G-beads through the GST moiety. The G-beads were then washed 3 times with 1 ml of 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl, and another 3 times with the

cleavage buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2.5 mM $CaCl_2$). As shown in Fig. 23A, the G-beads were able to extract GST-VP4 from the protein solution with minor contamination. The intact VP4 was released from the G-beads by protease digestion at room temperature with human thrombin (0.5 u/ml), which cleaved the junction sequence (LVRRGS) between GST and VP4. As shown in Fig. 23B, after thrombin digestion, the amount of GST-VP4 fusion protein (102 kDa) decreased and the amounts of VP4 (75 kDa) and GST (27 kDa) increased.

Expression of VP4 as 6xH-VP4 fusion protein

BTV-13 M1 cDNA was also cloned into the pQE30 (Qiagen) expression vector at the Sma I site using the procedures described previously. The plasmid carrying the correct orientation of BTV-13 M1 cDNA (pQE30BTV13M1) was transformed into to *E. coli* strain M15 (pREP4) (Qiagen) for expression. VP4 would be expressed as a fusion protein tagged with 6-histidines (6xH-VP4) in this system. Ten ml of the overnight culture grown at 37° C was diluted with growth media at a 1:20 dilution and allowed to grow for 3 hr. IPTG was then added to the culture at a final concentration of 2 mM to initiate the protein expression. As shown in Fig. 24, *E. coli* grown in different media had

different efficiencies of 6xH-VP4 protein expression. In the comparison of 2xYT (16 g bacto-tryptone, 10 g bacto-yeast extract, and 5 g NaCl per liter), Super Broth (25 g bacto-tryptone, 15 g bacto yeast extract, and 5 g NaCl per liter), and LB (10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl per liter), 2xYT gave the best yield in the expression of 6xH-VP4, and LB gave the lowest efficiency of expression. The 6xH-VP4 was also expressed as insoluble inclusion bodies. Lowering the growth and induction temperatures, and using different dilutions of *E. coli* culture and induction times did not change the formation of inclusion bodies. The expressed 6xH-VP4 was then purified in the denatured form using Talon^{TM} resin (Clontech), which is a non-nickel immobilized metal affinity resin, to purify recombinant 6xH proteins. The induced *E. coli* culture was harvested by centrifugation at 4,000 rpm for 10 min, and the pelleted cells were resuspended in 10 ml of STE (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA) buffer. The cells were lysed by sonication 5 times (30 sec each), and the lysed cells were then centrifuged at 4,000 rpm for 15 min to separate the soluble and insoluble fractions. The insoluble fraction containing 6xH-VP4 was dissolved in 10 ml of buffer containing 20 mM Tris-HCl, pH 8, 100 mM NaCl, and 6 M

guanidine-HCl for 10 min at room temperature. The protein solution was then centrifuged at 10,000x g for 10 min to remove any insoluble materials. The majority of 6xH-VP4 was found in the supernatant (data not shown). The supernatant containing the 6xH-VP4 was then mixed with 1 ml of Talon™ resin pre-equilibrated with the same buffer at room temperature for 20 min to extract the 6xH-VP4 protein. The resin was then washed with the same buffer for 10 min to remove unbound proteins. The washed resin was then packed into a 10-ml column. Elution buffer (20 mM Tris-HCl, pH 8, 100 mM NaCl, 8 M urea) containing different concentration of imidazole (5 ml of each concentration) was used to elute the 6xH-VP4, and 1-ml fractions were collected. As shown in Fig. 25, the 6xH-VP4 eluted at 40 mM imidazole.

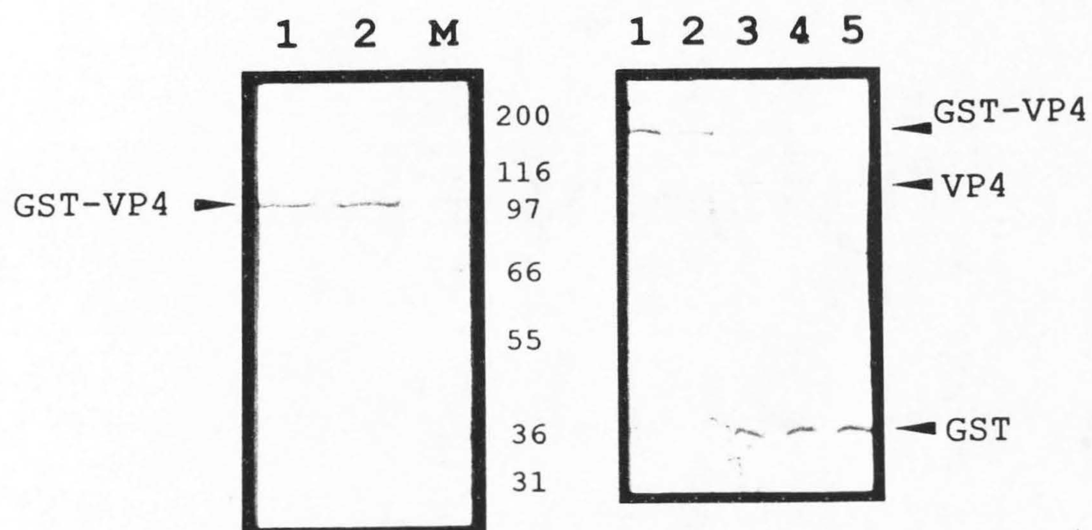

FIG. 24. SDS-PAGE analyses of purified GST-VP4 fusion proteins. (A) GST-VP4 fusion protein purified with glutathione-conjugated agarose beads in two preparations. Lane M: Molecular weight markers shown as kDa. (B) Thrombin cleavage of the purified GST-VP4 fusion protein. Lane 1: GST-VP4 fusion protein without thrombin; Lane 2: 10 min; Lane 3: 30 min; Lane 4: 60 min; Lane 5: 90 min incubation with thrombin at room temperature. The gels were stained with Coomassie blue.

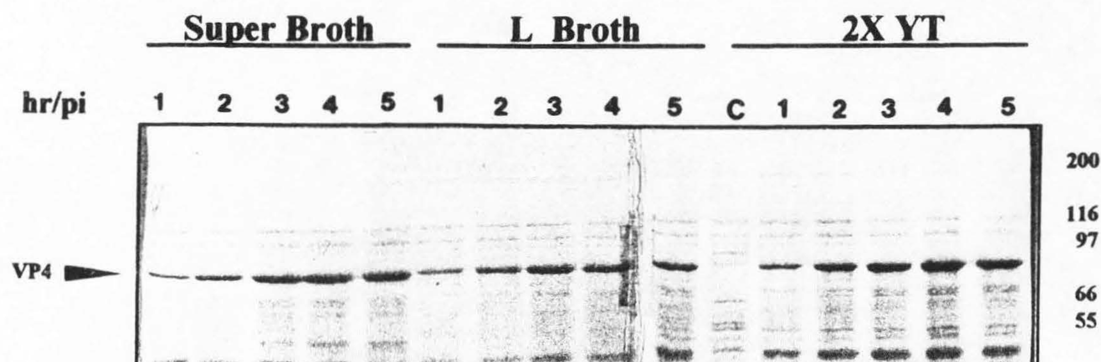

FIG. 25. SDS-PAGE analyses of the 6xH-VP4 fusion proteins expressed in different growth media. *E. coli* carrying pQE30BTV13M1 plasmid was grown in Super Broth, LB, and 2xYT media, respectively and induced with 2 mM IPTG for protein expression. The samples were analyzed on a 7.5% SDS-PAGE and stained with Coomassie blue. Numbers on top of the gel represent the hours post induction (hr/pi). Molecular weights were indicated on the right side of the figure (kDa).

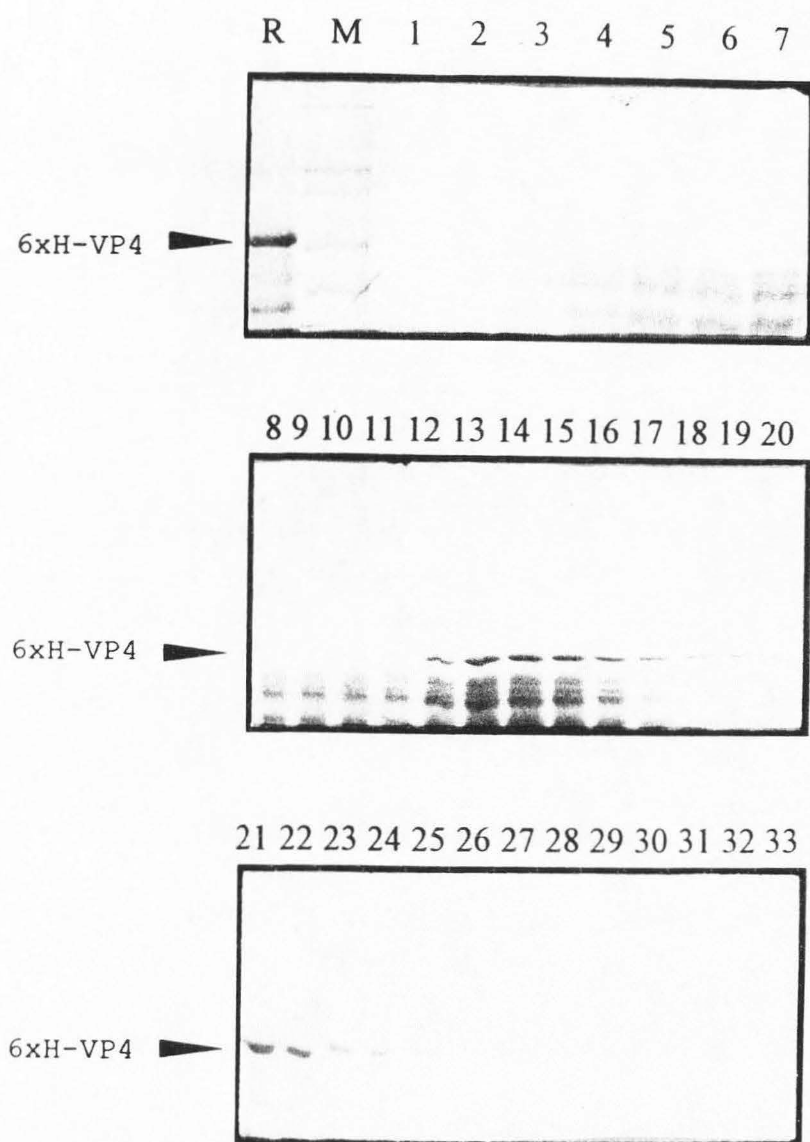

FIG. 26. Purification of 6xH-VP4 under denaturing conditions. Expressed 6xH-VP4 was extracted by Talon™ resin and eluted with 5 ml of 10 mM (Lanes 1 to 5), 20 mM (Lanes 6-10), 40 mM (Lanes 11 to 15), 80 mM (Lanes 16 to 20), 100 mM (Lanes 21-25), and 200 mM (Lanes 26 to 33) imidazole in the elution buffer. One-ml fractions were collected and 10 µl of each fraction were analyzed on 7.5% SDS-PAGE and stained with Coomassie blue. Lane M: Molecular weight markers (200, 116, 97, 66, and 55 kDa). Lane R: Talon™ resin with bound 6xH-VP4.

APPENDIX C.

COEXPRESSION OF VP4 AND VP6 IN INSECT CELLS

COEXPRESSION OF VP4 AND VP6 IN INSECT CELLS

MATERIALS, METHODS, AND RESULTS

Because VP4 that was expressed in both eukaryotic and prokaryotic cells was in the insoluble form, I investigated whether co-expressing it with another inner core protein would increase its solubility in insect cells. BTV-13 M1 and S3 (codes for VP6) cDNAs were cloned into the pAcUW51 (Invitrogen) baculovirus expression vector. The vector has two promoters (p10 and polyhedrin) to express two genes in the same insect cell. Full-length BTV-13 S3 cDNA was amplified as described in CHAPTER II using a pair of primer anchored with an EcoR I site (5'-GGATCCGAATTCGTTAAAA AATCGCATAT-3' and 5'-GGATCCGAATTCGTAAGTGTGAAATCGCC-3') and cloned into the vector pAcUW51 at the EcoR I site (pAcUW51BTV13S3). Two µg of the pAcUW51BTV13S3 plasmids were then digested with the BamH I restriction enzyme in 20 µl of reaction mixture. To fill in the BamH I digested sticky ends to generate blunt ends, 1 µl of each 0.5 mM dNTP and 5 units of Klenow were added to the mixture and incubated 15 min at 30° C. The blunt-ended plasmids were then purified by phenol/chloroform and ethanol precipitated as described in CHAPTER II. The BTV-13 M1 cDNA digested with Sma I (also blunt ended) was ligated to the blunt-

ended linear pAcuW51BTV13S3 plasmid to construct the pAcuW51BTV13S3-M1 plasmid. The plasmid construction was summarized in Fig. 26. The plasmid purification and co-transfection with linear viral DNA were according to the procedures described in CHAPTER III. As shown in lane T of Fig. 27, the recombinant baculovirus infected insect cells expressed both VP4 and VP6. However, the expressed VP4 was mainly in the insoluble portion (Lane P, Fig. 27), whereas VP6 was soluble (Lane S, Fig. 27). To investigate whether VP4 and VP6 formed a complex *in vivo*, co-immunoprecipitation was carried out with anti-VP6 MAbs (D34.5.7, D12.4.20, and H13.1). Insect cells infected with recombinant baculovirus were harvested after 72 hr post infection and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40). The lysed cells were centrifuged to separate the soluble and insoluble fractions. Fifty µl of each MAb were mixed with 250 µl of the supernatant, and incubated at 4° C for 1 hr. At the end of incubation, 50 µl of protein A-sepharose (50% v/v in PBS) were added to the solution and incubated at 4° C for 1 hr. The sepharose was then washed with the same buffer 3 times to remove the unbound antibodies and proteins. The protein-antibody complexes bound to the protein A-sepharose were solubilized by the SDS-PAGE sample buffer and analyzed by western blot

immunodetection using anti-VP4 or VP6 OAbs. As shown in Fig. 28, the VP6 protein was immunoprecipitated by anti-VP6 MAbs. However, VP4 did not co-precipitate with VP6. The result indicated that VP4 and VP6 did not form a complex *in vivo*. Similar results were obtained when the yeast two-hybrid system (Clontech) was used to investigate the interaction between VP4 and VP6 (data not shown).

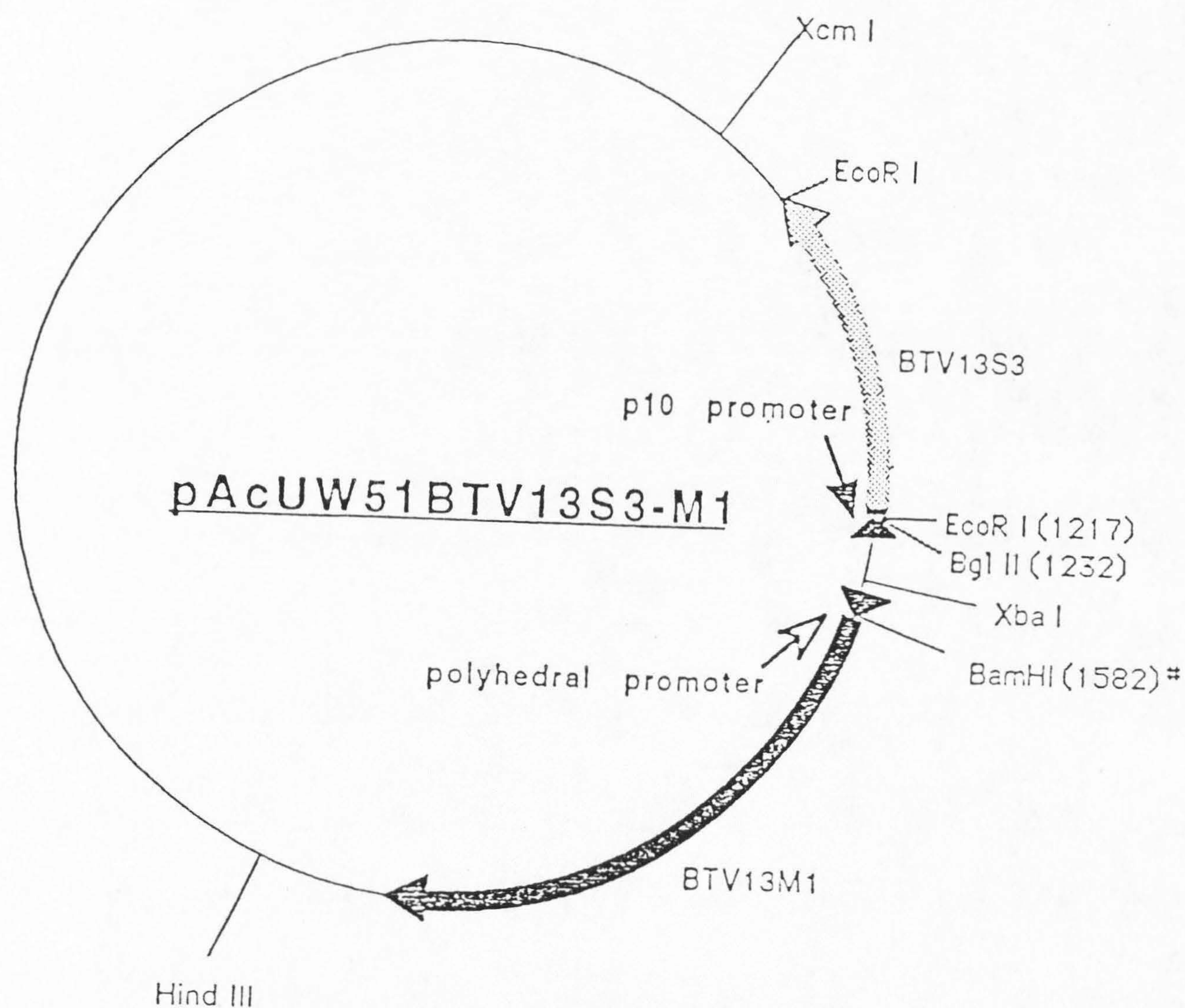

FIG. 27. Summary of the construction of pAcUW51BTV13S3-M1. BTV-13 S3 cDNA was cloned into the EcoR I site, and the expression of VP6 was controlled by the p10 promoter. BTV-13 M1 cDNA was cloned into the BamH I site, and the expression of VP4 was controlled by the polyhedrin promoter. #: the site of BamH I was destroyed by filling in the sticky ends to generate blunt ends.

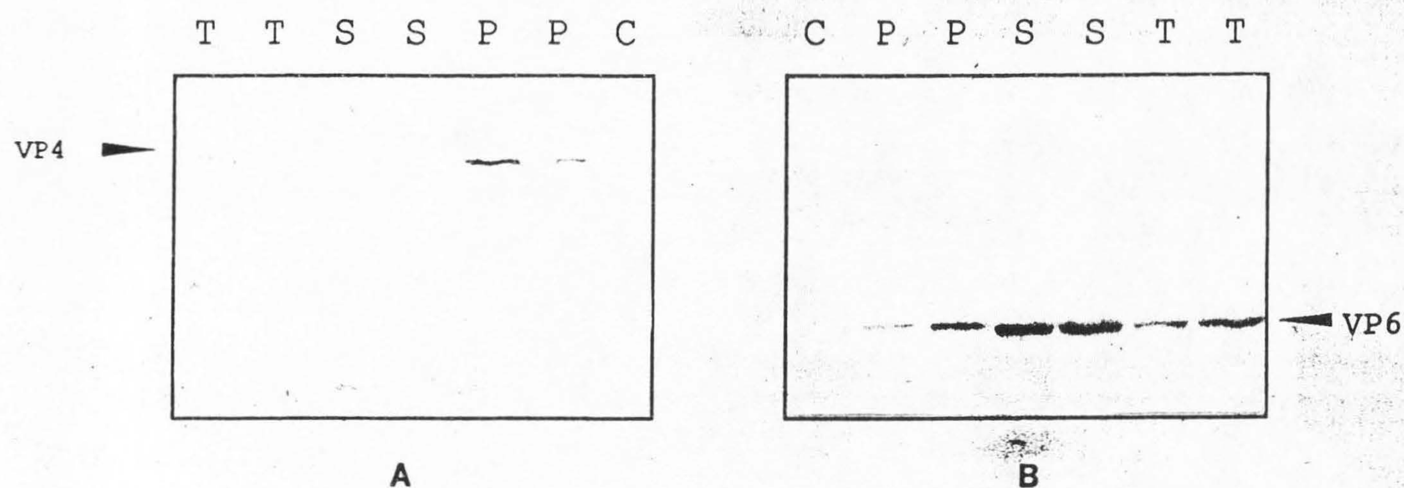

FIG. 28. Analysis of the coexpression of VP4 and VP6 in insect cells by western blot immunodetection. Insect cells infected with recombinant baculovirus carrying BTV-13 S3 and M1 cDNA were harvested, separated on SDS-PAGE, and transferred to nitrocellulose membranes as described in CHAPTER III. (A) Immunodetection with anti-VP4 OAb #82 (1:1000 dilution). (B) Immunodetection with anti-VP6 MAb (D34.5.7). Lane T: total cell lysate; Lane S: supernatant (soluble portion); Lane P: pellet (insoluble fraction). Duplicated samples were used for analysis.

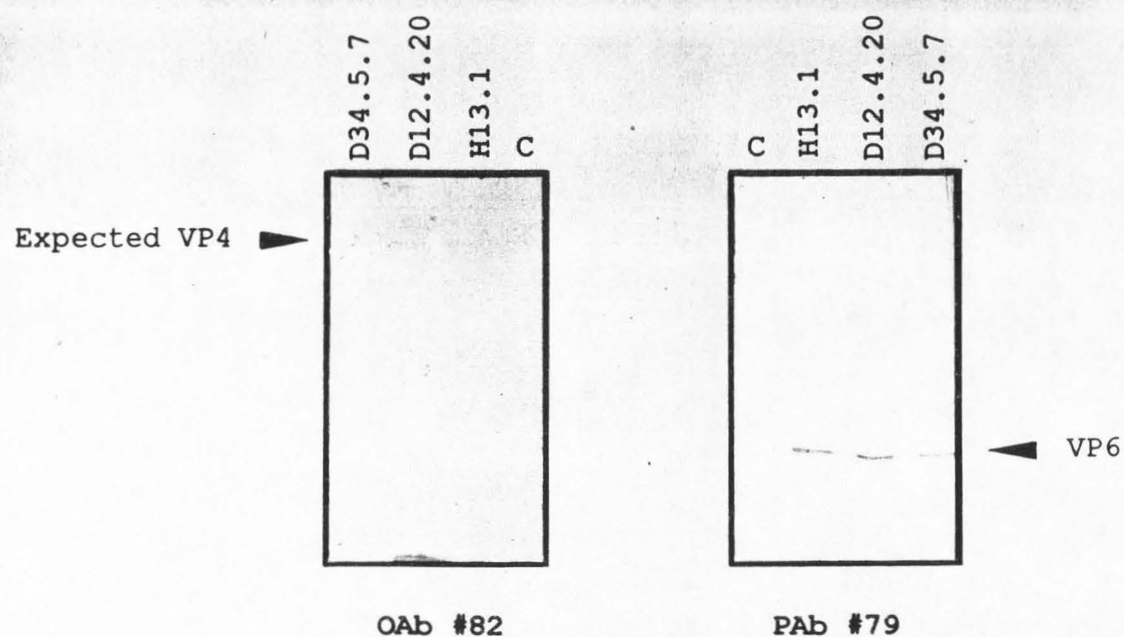

FIG. 29. Immunoprecipitation of insect cells coexpressing VP4 and VP6 proteins. The soluble fraction of the insect cell lysate (as described in Fig. 27) was immunoprecipitated with anti-VP6 MAbs (D34.5.7, D12.4.20, and H13.1). The immunoprecipitated complexes were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunodetected with anti-VP4 OAb #82 (1:1000 dilution) or anti-BTV virion PAb #79 (1:1000 dilution). The results indicated that VP4 and VP6 proteins were not co-precipitated with anti-VP6 MAbs. Lane C: mock-infected cell lysate.

APPENDIX D.

RESULTS OBTAINED USING DIFFERENT REAGENTS FOR PURIFYING VP4 FROM INSECT (SF-9) CELLS

TABLE 6

Results Obtained Using Different Reagents for Purifying VP4 from Insect (SF-9) Cells

Lysis buffers (sonication)	Solubilization buffers	Solubility* (1-5 scale)	Buffer for dialysis	GTP binding activity
1% NP40, 150 mM $MgCl_2$, 50 mM Tris, pH 8.	200 mM $MgCl_2$, 10 mM DTT, 50 mM Tris, pH 8	1		
1% NP40, 1mM EDTA, 50mM Tris, pH 7.5	1.5% N-Lauroyl Sarcosine , 50 mM Tris, pH 8	5	PBS-**ppt**; 50mMTris pH 8.8- **ppt**	No (No RNA binding, either).
Same as above	100 to 500 mM EDTA, 50mM Tris, pH 8	2	10 mM Tris, pH 8- **ppt**	Yes (dot blot)
Same as above	8 M urea (room temperature)	2.5	10 mM Tris, pH 7.5-**ppt**	No
Same as above	8 M urea, 10 or 50 mM DTT (at room temperature)	2.5		
Same as above	6 M Guanidine-HCl	3.5	10 mM Tris, pH 7.5-**ppt**	No
1% NP40, 10 mM EDTA, 50 mM HEPES, pH 7.3	100 mM EDTA	1.5		
Same as above	1% Tw-20 in lysis buffer	0		
Same as above	1% Chaps in lysis buffer	0		
1% NP40, 10mM EDTA, 50 mM Tris, pH 8.8	100 and 500 mM EDTA, pH 9	1.5		
Same as above	1% Tw-20 in PBS	0		
Same as above	1%DOC in PBS	1		No
1% NP40, 0.2 M NaCl, 10mM EDTA, 50mM Tris, pH 8		0		
0.01% NP40, 150 to 500 mM NaCl, or 150 mM $MgCl_2$	0.001 to 0.1% NP40	0		Yes (supernatant of lysis buffer)
0.2 M NaCl, 10mM EDTA, 50mM Tris, pH 8		VP4 was detected in supernatant by OAb		

ppt: The protein precipitated during the dialysis.

* Scale 5= soluble and scale 0= insoluble.

APPENDIX E.

PERMISSION-TO-REPRINT LETTERS

TO: Virology Editor
525 B Street Suite 1900,
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MAR 4 1996

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FROM: Huang, I-Jen
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REMARKS: ☐ Urgent ☐ For your review ☐ Reply ASAP ☐ Please Comment

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Huang, I-J., Hayama, E., Jenog, Y.-J., and Li, J.K.-K. (1993). Conservation of the segment 4 gene sequences and of a leucine zipper motif in VP4 among five US bluetongue viruses. Virology 195, 772-779.

Huang, I-J., Hwang, G.-Y., Yang, Y.-Y., Hayama, E., and Li, J.K.-K. (1995). Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five U.S. bluetongue viruses. Virology 214, 280-288.

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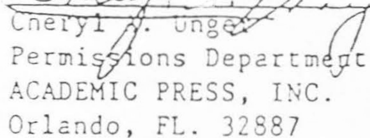

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Sincerely yours

Yi-Yuan Yang

Yi-Yuan Yang, Ph.D.
Department of Medical Technology
#250, Wu-Hsing St.
Taipei Medical College
Taipei, Taiwan

Dear Bruce,
Here is the release letter from author on the papers which were shared with Mr. Bruce I-Jen Huang. I, Guang-Yuh Hwang, give my permission to Mr. Huang to include the information from the papers into part of his dissertation. The shared papers were listed below:
Yang, Y.Y., Chiou, J.F., Hwang, G.Y., Huang, I-J., and Li, J.K.K. 1992. Evolutionary analysis of United State bluetongue viruses using the cognate S2 genes. Virus Res.25:241-249. (Elsevier Science Publishers B.V.)
Huang,I-J., Hwang, G.Y., Yang, Y.-Y., Hayama, E., and Li, J.K.K.1995. Sequence analyses and antigenic epitope Mapping of the putative RNA-directed RNA polymerase of five US bluetongue viruses. Virology 214, 280-288. (Academic Press, Inc.)

Best wishes,

Guang-Yuh Hwang

1. Conservation of the segment 4 gene sequence and of a leucine zipper motif in VP4 among five US bluetongue viruses. 1993 Virology
2. Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five US Bluetongue viruses. 1995 Virology

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Date: February 23. 1996

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Education:

Ph.D. :Biology/Molecular Biology. Utah State University, Logan, Utah, USA; 1996
M.S. :Biology/Plant Physiology. Utah State University, Logan, Utah, USA; 1991.
Diploma :Chemical Engineering. Min-Chi Institute of Technology, Taipei, Taiwan; 1982.

Membership in Professional Societies:

Member of Society of Chinese Bioscientists in America.
Member of American Society for Microbiology.
Member of American society for Virology.

Research Experience:

* Synthesized cDNA using viral dsRNA of bluetongue virus or DNA as templates.
* Cloned and sequenced S2, M1, and L1 genes of bluetongue viruses.
* Expressed bluetongue viral protein NS2, VP6, VP4, VP1 and rotaviral VP7 in baculovirus expression system; expressed bluetongue viral NS2 and VP4 in prokaryotic system including pGEX, pTrxFus, and pQE expression vectors; expressed bluetongue viral VP4 in yeast (*Pichia pastoris*).
* Purified proteins from plant tissue (leaves and roots) and recombinant proteins from insect cells and *E.coli.*
* Produced both polyclonal and oligoclonal antibodies in rabbits against plant (ferredoxin) and viral proteins (NS2, VP4, and VP1 of bluetongue viruses).
* Mapped antigenic epitopes of bluetongue viral NS2, VP4, and VP1 proteins.

* Mapped nucleic acid binding domains of bluetongue viral VP4 protein.
* Identified the myristylation of bluetongue viral VP7 protein.
* Phylogenetic analyses of bluetongue viruses based on both nucleic acids and deduced amino acids sequences.

Technical Experience:

* Gel electrophoresis: polyacrylamide, agarose, and two-dimensional.
* Blotting: Western, Southern, Northern, Northwestern, and dot blots.
* Polymerase chain reaction (PCR) and cloning.
* DNA sequencing: manual dideoxynucleotide termination method.
* *In vitro* transcription and translation.
* Chemically cross linking proteins.
* Virus purification through sucrose gradient.
* Tissue culture: BHK-21, L-, and insect cells.
* *In vivo* labeling of proteins in tissue culture.
* Protein expression: pGEX, pTrxFus, and pQE expression vectors in *E.coli*; pHIL expression vector in yeast; baculovirus in SF-9 and High-5 cells.
* Site-directed mutagenesis.
* Protein purification: ion exchanger, affinity, and sizing columns.
* Antibodies production in rabbits.
* Electrophoretic mobility shift assay.
* Yeast two-hybrid system.
* Wisconsin sequence analysis package (GCG) software for analyzing both nucleic acids and proteins.
* Green house; hydroponics.

Awards:

First place of Student paper competition in 1995 annual meeting of American Society for Microbiology Intermountain Branch at Idaho State University, Idaho.

Student travel grant from American Society of Virology; 1994 ASV 13th annual meeting at the University of Wisconsin-Madison, Wisconsin.

Teaching Experience:

1995: Teaching assistant for Biology 674: Molecular Biology Laboratory under Dr. Joseph K.-K. Li.

1994: Teaching assistant for graduate level Molecular Biology Laboratory under Dr. Joseph K.-K. Li.

1990: Teaching assistant for Mineral Nutrition Laboratory under Dr. Gene W. Miller.

Publications:

1. **Huang, I-J.** (1991). Study of ferredoxin and flavodoxin from tobacco leaves and roots under normal and iron-stressed condition. Master thesis (79 pages). Utah State University, Logan, Utah, USA.
2. **Huang, I-J.**, Welkie, G.W., and Miller, G.W. (1992). Ferredoxin and flavodoxin analysis in tobacco in response to iron stress. J. Plant Nutrition 15, 1765-1782.
3. Yang, Y.-Y., Hwang, G.-Y., Chiou, J.-F., **Huang, I-J.**, and Li, J.K.-K. (1992). Evolutionary analysis of five US bluetongue viruses using the cognate S2 genes. Virus Res. 25, 241-249.
4. **Huang, I-J.**, Hayama, E., Jeong, Y.-J., and Li, J.K.-K. (1993). Conservation of the segment 4 gene sequence and of a leucine zipper motif in VP4 among five US bluetongue viruses. Virology 195, 772-779.
5. Miller, G.W., **Huang, I-J.**, Welkie, G.W., and Pushnik, J.C. (1995). Function of iron in plants with special emphasis on chloroplasts and photosynthetic activity. *In*: Iron Nutrition in Soils and Plants. pp 19-28. ed. J. Abadia, Kluwer Academic Publisher, Netherlands.
6. **Huang, I-J.**, Hwang, G.-Y., Yang, Y.-Y., Hayama, E., and Li, J.K-K. (1995). Sequence analyses and mapping of the putative RNA-directed RNA polymerase of five US bluetongue viruses. Virology 214,280-288.
7. **Huang, I-J.** and Li, J.K.-K. (1996). Delineation of the antigenic determinant and ssRNA-binding domains of the multimeric bluetongue virus inner core protein VP4. (manuscript in preparation).
8. **Huang, I-J.** and Li, J.K.-K. (1996). Expression and purification of bluetongue virus guanylyltransferase. (manuscript in preparation).

Abstracts:

1. **Huang, I-J.**, Welkie, G.W., and Miller, G.W. (1991). Ferredoxin and flavodoxin analysis in tobacco in iron

stress. The sixth international symposium on iron nutrition and interaction in plants, Logan, Utah, USA.

2.Yang, Y.-Y., Chiou, J.-F., Hwang, G.-Y., **Huang, I-J.**, and Li, J.K.-K. (1992). Evolution of bluetongue viruses in Northern America. The fourth SCBA international symposium, Singapore University, Singapore.

3.**Huang, I-J.**, Hayama, E., Jeong, Y.-J., and Li, J.K.-K. (1992). Comparative sequence analysis of segment 4 genes of five US bluetongue viruses. The fourth SCBA international symposium, Singapore University, Singapore.

4.Hwang, G.-Y., Li, J.K.-K., Yang, Y.-Y., **Huang, I-J.**, Hayama, E., and Chiou, J.-F. (1992). Molecular phylogenetic analysis of bluetongue viruses using cognate gene sequences representing half of the viral genome. Fourth international symposium on double-stranded RNA viruses. Scottsdale, Arizona, USA.

5.**Huang, I-J.**, Hayama, E., and Li, J.K.-K. (1993). Analysis of cognate gene sequence, conserved leucine zipper motif and antigenic epitopes on VP4 protein of five US bluetongue viruses. 12th international symposium of American Society of Virology. University of California, Davis, CA., USA.

6.Lin, H., **Huang, I-J.**, and Li, J.K.-K. (1994). Expression and purification of the nonstructural protein 2 (NS2) of the US bluetongue virus serotype 17. Intermountain branch meeting of American Society for Microbiology. Brigham Young University, Provo, Ut., USA.

7.**Huang, I-J.** and Li, J.K.-K. (1994). Identification of the antigenic epitopes of US Bluetongue virus VP4 proteins. 13th international symposium of American Society of Virology. University of Wisconsin-Madison, Madison, WI., USA.

8.Li, J.K.-K., Hayama, E., **Huang, I-J.**, Hwang, G.-Y., Yang, Y.-Y., and Lin, H. (1994). New paradigm in bluetongue viruses: from molecular phylogeny to antigenic epitopes and functional domains. First APSB international symposium and workshop. August 15-18, Singapore.

9.Li, J.K.-K., **Huang, I-J.**, and Hayama, E. (1994). Role of RNA/protein interaction in the packaging of the ten bluetongue virus double-stranded RNAs into the virion. Third Asia-Pacific Congress of Medical Virology. October 23-28, Beijing, China.

10. Li, J.K.-K., Hwang, G.-Y., **Huang, I-J.**, Hayama E., and Yang, Y.-Y. (1995). Molecular phylogenetic analyses of five US bluetongue viruses using the entire genomic sequence of the L1 segment which encodes the putative RNA-dependent RNA polymerase. Fifth international symposium on double-stranded RNA viruses. March 19-23, Jerba, Tunisia.

11. Lin, H., **Huang, I-J.**, and Li, J.K.-K. (1995). Characterization of the Single Stranded RNA Binding Domains of the Non-Structural protein 2 (NS-2) of the US Bluetongue Virus Serotype 17. American Society for Microbiology Intermountain Branch Annual Meeting, April 22, Idaho State University, Pocatello, Idaho, USA.

12. **Huang, I-J.** and Li, J.K.-K. (1995). Mapping of Nucleic Acid Binding Domain of the VP4 of Bluetongue Virus. American Society for Microbiology Intermountain Branch Annual Meeting, April 22, Idaho State University, Pocatello, Idaho, USA.

13. Li, J.K.-K., Hwang, G.-Y., Yang, Y.-Y., Hayama, E., and **Huang, I-J.** (1995). Molecular phylogenetic analyses of bluetongue viruses. Sixth SCBA international symposium. June 24-29, Vancouver, B.C., Canada.

14. Lin, H., **Huang, I-J.**, and Li, J.K.-K. (1995). Expression, Purification, and Characterization of the Non-Structural Protein (NS-2) of the US Bluetongue Virus Serotype 17. 14th international symposium of American Society of Virology. University of Texas, Austin, Texas., USA.

15. Li, J.K.-K., **Huang, I-J.**, Hayama, E., and Lin, H. (1996). Structure and functions of ss-RNA binding proteins of bluetongue viruses. The second APSB international symposium and workshop. July 6-12, Hong Kong University of Science and Technology, Hong Kong.

16. **Huang, I-J.**, Mayes, R., and Li, J.K.-K. (1996). Characterization of the surface-accessible antigenic determinants and ss-RNA binding domains on the VP4 of bluetongue virus. 15th international symposium of American Society of Virology. July 13-17, University of Western Ontario, Canada.