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BLUETONGUE VIRUS EVOLUTION:

SEQUENCE ANALYSES OF THE GENE CODING

FOR THE MAJOR SEROGROUP ANTIGEN

by

Timothy F. Kowalik

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

of

DOCTOR OF PHILOSOPHY

in

Biology

Approved:

Major Professor	Committee Member
Committee Member	Committee Member
Committee Member	Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah
1989

in loving memory of

Mary Stromko and Bertha Powell

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ABSTRACT

BLUETONGUE VIRUS EVOLUTION: SEQUENCE ANALYSES of the GENE CODING for the MAJOR SEROGROUP ANTIGEN

by

Timothy F. Kowalik, Doctor of Philosophy

Utah State University, 1989

Major Professor: Dr. Joseph K.-K. Li
Department: Biology

A study was undertaken to better understand the genetic relationship of five United States prototype bluetongue virus serotypes. Genomic double-stranded RNA segment S1, which encodes the major core protein and serogroup antigen, VP7, was used as a marker gene for the sequence analyses. The S1 segments from BTV-2, 11, 13, and 17 were cloned and sequenced by methods developed during the course of this investigation. These results were compared with previously published sequence data from segment S1 of BTV-10. The S1 segments are 1156 base pairs long and contain a common open reading frame capable of coding for a protein (VP7) of 349 amino acids. This reading frame is flanked by an 18 base 5' noncoding stretch and an 89 base 3' noncoding region. Both termini are well conserved among the serotypes. The 3' end of each serotype has the potential to form a stable stem-loop structure. VP7 is very well conserved among the

five serotypes. It has a predicted charge of +1 to +1.5 at pH 7.0. Hydropathy and secondary structure predictions show an alternating pattern of hydrophobic and hydrophilic amino acid regions. Based on this data, an eight-stranded ß-barrel is predicted for VP7. Proteins of similar function in single-stranded RNA viruses have this same structure. This observation suggests a common origin of bluetongue viruses with single-stranded RNA viruses. The ß-barrel may also serve as a target for antiviral drugs as is the case with picornaviruses. Phylogenetic analyses show that BTV-2, 10, 11, and 17 are closely related and that BTV-13 is the most distant of the five United States serotypes. Similar comparisons of the previously published L2 sequence show a similar pattern. By using the 29-year span between the appearance of BTV-10 and BTV-2 in the United States, an evolutionary rate of 2.2×10^{-3} nucleotide substitutions/site/year was estimated. This is similar to the gag gene of retroviruses and the RNA polymerase gene of influenza. A rate of this magnitude is evidence that double-stranded RNA viruses, like single-stranded RNA viruses, do not have polymerase proofreading activity. (139 pages)

CHAPTER I

LITERATURE REVIEW AND PROJECT OUTLINE

INTRODUCTION

Bluetongue virus classification

Gomatos et al. (1962) first demonstrated that reovirus particles contained double-stranded RNA (dsRNA). The next year, several virus species from both plants and animals were shown to also contain dsRNA (Gomatos and Tamm, 1963). This was followed by identification of dsRNA in bluetongue virus (Verwoerd, 1969) and rotavirus (Welch, 1971). Originally, all viruses containing dsRNA were grouped into the Diplornaviridae family (Verwoerd, 1970). However, this proved unfeasible, as there were major differences in morphology and replication strategies among the viruses (Joklik, 1983). At present, the Reoviridae family is composed of six genera with BTV belonging to Orbivirus.

The term, Orbivirus, was originally coined to describe a group of viruses spread by arthropods and having common morphological features (Borden et al., 1971). The genus name is a derivative of the Latin orbis, meaning ring or circle. Negatively stained virus in this genus appear "doughnut-shaped" when viewed by electron microscopy. Viruses in this genus differ from another group of arthropod-borne viruses, the Arbovirus, by the resistances of orbiviruses to solvents and detergents because they lack a lipid envelope (Gorman et al., 1983). Borden et al.

(1971) identified ten serogroups within this genus (there are now twelve), of which BTV is the prototype.

Disease and epidemiology

BTV is the causative agent of bluetongue, a degenerative and at times fatal disease of ruminants such as sheep, cattle, goats, deer, and antelope (Davies and Walker, 1974; Fulton et al., 1981; Hoff et al., 1973; Hoff and Hoff, 1976; Inverso et al., 1980). Sheep experimentally infected with BTV usually present with a febrile reaction and a viremia within thirty days of infection (Luedke, 1969; Jeggo et al., 1983). Disease symptoms become much more severe, involving a hypersensitivity reaction upon subsequent challenges with BTV (Luedke, 1969; Stott et al., 1985; Mahrt and Osburn, 1986a). This may be the result of an IgE-mediated (type I) hyperimmune response (Anderson et al., 1987). Cattle usually show no clinical symptoms other than a viremia which may become chronic or latent (Dutoit, 1962; Bowne 1971; Luedke et al., 1970; Luedke et al., 1977b).

Bluetongue disease is manifested by the appearance of lesions in the vascular endothelium, one of the sites of BTV replication. The secretion of exudates and the development of additional lesions nearby result in blockage of blood vessels and localized hypoxia (Thomas and Neitz, 1947). Similar lesions are observed in the mucosa of the upper digestive tract along with excessive salivation

(Stott et al., 1985; Mahrt and Osburn, 1986b). Death is apparently the result of congestive heart failure (Marht and Osburn, 1986b). BTV has also been associated with congenital malformations, fetal death, and abortion (Osburn et al., 1971; Luedke et al., 1977a).

The virus is transmitted by *Culicoides* species (Dutoit, 1944; Bowne and Jones, 1968). However, BTV has been found in the semen of latently infected bulls (Breckon et al., 1980). Although attempts to directly demonstrate vertical transmission of BTV in cattle have been unsuccessful (Bowne et al., 1983), the virus was isolated from twin newborn North American elks whose mother had an ongoing BTV infection (Stott et al., 1982).

BTV was originally isolated in Africa and restricted to that continent for 40 years before spreading to the Middle East (Dutoit, 1944; Bowne, 1971; Gorman et al., 1983). BTV is presently found worldwide in temperate and tropical regions although it is usually observed as endemic episodes (Bowne, 1971). At present, there are at least 24 serotypes known throughout the world (Gorman et al., 1983).

BTV is of economic importance in the endemic areas of the United States, Australia, Africa, and the Middle East. This is the result of restrictions on the export of ruminants and their products (semen and ova) from these areas. Control of the disease is difficult due to the often subclinical nature of bluetongue and the lack of rapid diagnosis. BTV is usually found in samples by

standard serological means which identify a common BTV antigen (Hubschle et al., 1980). Serotyping is accomplished by plaque neutralization with hyperimmune guinea pig sera (Howell and Verwoerd, 1971) or by a much less reliable ELISA method (Hubschle et al., 1980).

There is no cure or therapeutic regimen for the treatment of BTV infections. An attenuated vaccine based on several South African BTV serotypes has been in use in that country for some time despite evidence that it is dangerous (viral reactivation and hyperimmune responses) and not very effective (Bowne, 1971). Attempts to immunize sheep with chemically inactivated virus have proven unsuccessful and have resulted in the hypersensitization of the animals (Stott et al., 1985; Mahrt and Osburn, 1986a; 1986b). Standard means to control viruses have proven unsuccessful when applied to BTV. Therefore, a better understanding of the molecular biology and life cycle of BTV seems necessary for the development of novel means to control and eventually eradicate the virus. Since BTV serves as a model, the studies undertaken here will provide a comparative system for other members of Orbivirus.

Virus structure

Electron microscopic studies of BTV showed that negatively stained virus particles have a diameter of 53 nm and no evidence of a viral envelope structure. The virus was determined to be icosahedral, having 92 capsomers and a

structure very similar to reoviruses (Studdart et al., 1966). However more recent studies have shown BTV to have a poorly defined outer structure with a mean diameter of 65.5 nm and a well-defined core structure of 55 to 58 nm. The core contains 32 symmetrically arranged capsomers and a 5:3:2 symmetry (Bowne and Ritchie 1970; Els and Verwoerd, 1969; Verwoerd et al., 1972; Mertens et al., 1984; 1987a; Eaton et al., 1987; Huismans et al., 1987b). It appears that Studdart et al. (1966) were describing the viral cores. These studies demonstrated that a complete BTV virion is composed of two structures, a diffuse outer capsid surrounding a well-defined core. The outer capsid of BTV is composed of two proteins and is free of lipids (Verwoerd, 1969). A very recent study demonstrated that an unusual budding event by BTV does occasionally result in enveloped virions (Eaton, unpublished observations). The core particle is composed of genomic dsRNA and five proteins (Verwoerd, 1969).

In 1969, BTV was first purified and shown to have a genome composed of dsRNA (Verwoerd, 1969). The BTV genome was subsequently shown by polyacrylamide gel electrophoresis (PAGE) to be composed of ten discrete segments of dsRNA (Verwoerd et al., 1972). The total size of the genome was calculated to be 12×10^6 Da when compared to the 15×10^6 Da estimate for reovirus.

Virus replication

BTV replication takes place in the cytosol. Studies by Lecatsas (1968) and Cromack et al. (1971) show virus absorbed to cytoplasmic membranes within minutes. This is followed by the appearance of BTV inside of phagocytic vesicles. At 1 hour post infection (p.i.), the outer capsid (VP2 and VP5) of the virus disappears and only cores containing VP1, VP3, VP4, VP6, and VP7 remain (Huismans et al., 1987b). This uncoating activates a viral RNA polymerase activity which results in the accumulation of virally-encoded mRNAs in the cytoplasm. From 6 hours p.i., the core particles are converted into a different structure with a diameter of 40 nm. These particles have lost the main structural component of the core (VP7) and now contain VP1, VP3, VP4, and VP6. They are relatively unstable in low salt and no longer retain the transcriptase activity of the core (Huismans et al., 1987b). By 8 hours p.i., dense viral inclusion bodies (VIB) and fibrillar networks of virally encoded tubules (NS1) are present in the cytosol (Lecatsas, 1968; Huismans and Els, 1979). These structures as well as complete progeny virions are all associated with cellular filaments composed of vimentin (Eaton et al., 1987). It has been believed that the VIBs are the primary site of virus morphogenesis (Gorman et al., 1983; Hyatt and Eaton, 1988). Recently, Eaton (unpublished observations) has provided evidence for the morphogenesis of cores or core-like structures in the VIB, while further maturation

and the addition of the outer capsid takes place on or near the tubules. Virus release appears to be the result of a budding mechanism that usually does not result in the virus particle being enveloped (Eaton et al., 1988; Eaton, unpublished observations).

Virus genome organization

The genome of BTV was demonstrated to be dsRNA by its resistance to ribonucleases, sensitivity to alkali, and the production of a sharp hyperchromic shift upon heating (Verwoerd, 1970). The genome is composed of ten discrete segments of dsRNA and does not appear to contain the very small molecular weight RNAs that are observed in reovirus (Verwoerd, 1970; Bellamy et al., 1972). The ten dsRNA segments can be separated by electrophoresis through polyacrylamide or Nusieve agarose gels (Verwoerd, 1970; Gorman et al., 1981; Kowalik and Li, 1987). In either system, the segments migrate in three groups: three large segments (L1, L2, L3), three medium segments (M1, M2, M3), and four small segments (S1, S2, S3, and S4). The nomenclature which will be used here is based upon the relative migration of the dsRNA segments through agarose gels in the presence of an electrical field (Kowalik and Li, 1987; Mertens et al., 1987b).

Recently, the entire genome of United States prototype BTV serotype 10 (BTV-10) was sequenced (Roy, 1989). The genome of BTV was shown to consist of 19,218 bp of dsRNA.

The sizes of the individual segments range from 3954 bp (L1) to 822 bp (S4; Roy, 1989). Each BTV segment codes for at least one known protein and all of the gene-coding assignments discussed below have been verified by both in vivo pulse labelling experiments (Martin and Zweerink, 1972; Verwoerd et al., 1972; Huismans, 1979) and in vitro translation studies (Grubman et al., 1983; Sangar and Mertens, 1983; Mertens et al., 1984; Van Dijk and Huismans, 1988). In some cases, the sequence of a specific dsRNA segment from more than one BTV serotype has been determined.

Segment L1 (3954 bp) encodes a minor core protein, VP1. L1 has been sequenced and examination of the deduced amino acid sequence shows that VP1 has a predicted MW of 144,000 and that VP1 may be a virallyencoded RNA polymerase (Roy et al., 1988). Recently, VP1 was expressed in a baculovirus system and shown to have RNA polymerase activity (Urakawa et al., 1989). The L1 segment is the only BTV dsRNA segment known to not use the first AUG codon in the 5' end of the sequence (Rao et al., 1983b; Mertens and Sangar, 1985).

Segment L2 (2926 bp) encodes VP2, one of the two outer capsid proteins. VP2 has a predicted MW of 111,000 and is the main, if not the only, serotype-specific antigen (Huismans and Erasmus, 1981; Appleton and Letchworth, 1983; Grubman et al., 1983; Purdy et al., 1985; Ghiasi et al., 1987; Inumura and Roy, 1987; Yamaguchi et al., 1988a;

Mertens _et al._, 1989). Since serotyping is dependent upon viral neutralization, it has been inferred but not demonstrated that VP2 is the cell attachment protein (Verwoerd _et al._ 1972; Huismans and Erasmus, 1981). However, very recent data show that the other outer capsid protein, VP5, plays a role in the determination of serotype (Mertens _et al._, 1989). VP2 is also the viral hemagglutinin (Cowley and Gorman, 1987).

The L3 gene segment (2772 bp) codes for VP3, one of the two major core proteins of BTV. Sequence analysis of segment L3 shows that VP3 has a MW of 103,000 (Purdy _et al._, 1984; Ghiasi _et al._, 1985; Gould, 1987). As a main structural protein, VP3 has a relatively low number of charged amino acids.

Segment M1 (2011 bp) codes for VP4 and is a minor component of the inner core. The protein has a size of 76,000 Da and has high content of charged amino acids (Yu _et al._, 1987). No function(s) has been assigned to VP4.

Segment M2 (1769 bp) encodes NS1, the nonstructural, tubule-forming protein (Lecatsas, 1968; Huismans and Els, 1979; Lee and Roy, 1987). The tubules are also formed independent of BTV infection as shown by the expression of M2 in _Spodoptera frugiperda_ cells (Urakawa and Roy, 1988). NS1 has a MW of 65,000 and is the most highly expressed BTV protein both _in vivo_ and _in vitro_. The function of NS1 is not clear, but it is somehow involved in transforming viral cores produced in the VIB into mature virions. These

tubules may also play a role in transport of virus to the cell surface (Eaton, unpublished observations).

Segment M3 (1638 bp) codes for the other outer capsid protein, VP5. This protein is 59,000 Da and is rich in the nonpolar amino acids, alanine and isoleucine (Purdy et al, 1986; Gould and Pritchard, 1988; Wade-Evans et al., 1988). The function of VP5 is not well characterized, but it has been demonstrated to play a role in defining the serotype (Mertens et al., 1989).

Segment S1 (1156 bp) encodes VP7, the major core protein and group-specific antigen of BTV (Huismans and Erasmus, 1981). VP7 is approximately 38,000 Da and not very hydrophobic for a structural protein (Yu et al., 1988; Kowalik and Li, 1989). Although the biological significance of this observation is unknown, VP7 only contains a single lysine residue. This is unusual for a virus core protein (Kowalik and Li, 1989). There is also some preliminary immunological evidence that VP7 may be exposed to the exterior surface of the viral outer capsid (Eaton, unpublished observations).

S2 codes (1121 bp) for another nonstructural protein, NS2. This protein has a MW of 49,000 and is the only BTV protein known to be phosphorylated (Huismans et al., 1987a; Devaney et al., 1988; Fukusho et al., 1989; Hall et al., 1989). NS2 has a high number of cysteines and is rich in hydrophilic amino acids. The protein has the ability to bind ssRNA and it has been hypothesized that NS2/RNA

complexes may serve as nucleation points for BTV maturation (Huismans et al., 1987a; Kowalik and Li, 1989).

Segment S3 (1046 bp) encodes VP6, a minor component of the virus core. VP6 has a MW of 36,000 and carries a strong positive charge (+11; Fukusho et al., 1989). It has a high content of glycine residues (12% of the total amino acid number) and only a single cysteine. Although a function has not been assigned to VP6, it has been recently shown to possess an affinity for BTV dsRNA when expressed in a baculovirus system (Roy, unpublished observations). Huismans et al. (1987a), mention that a nonstructural protein of 16,000 Da (NS4) whose function is not known is also expressed from segment S3.

Segment S4 (822 bp) of BTV encodes NS3 and NS3a, the latter of which is a truncated version of the former (French and Roy, 1989; Kowalik and Li, 1989). NS3 is 26,000 daltons and has a relatively hydrophilic nature (Lee and Roy, 1986; Gould, 1988). No function(s) has been assigned to NS3/NS3a.

Genetic relationships

The segmented nature of the BTV genome offers the potential of the individual genome segments to reassort with different BTV serotypes and other members of Reoviridae. Reassorting of genome segments has been demonstrated among BTV serotypes in tissue culture, vertebrate hosts and the Culicoides insect vectors

(Sugiyama et al., 1981; Kahlon et al., 1983; Rao and Roy, 1983; Samal et al. 1987; Stott et al., 1987). The insect system has a higher reassortment frequency than the vertebrate system (Samal et al., 1987). An attempt to reassort BTV genomic segments with other closely related orbiviruses was unsuccessful (Brown et al., 1988). Genome reassorting within the BTV serogroup and genetic drift provide two mechanisms for BTV to evolve. It may also be possible for dsRNA viruses to change by recombination. An example of a duplication and possible recombination event has been described for rotavirus (Gorziglia et al., 1989). However, there is no evidence in the literature of such an occurrence in BTV.

The genetic relationships among the many BTV serotypes have been examined by different approaches. Huismans and Howell (1973) analyzed the BTV serotypes found in South Africa by RNA/RNA reassociation. They found that most of the segments were very well conserved with the two dsRNA segments (L2 and M3) coding for the outer capsid proteins (VP2 and VP5) being the most variable.

RNA fingerprinting studies were done to examine the relationship among the four BTV serotypes (BTV-10, 11, 13, and 17) present in the United States at that time (Sugiyama et al., 1981; 1982; Rao et al., 1983a). These experiments showed that segments L2, L3, M3 and S1 were variable. Based on these results, it was concluded that BTV-17 evolved from BTV-11, BTV-10 and 11 shared a common gene

pool, and BTV-13 had very little similarity with the other three serotypes.

When compared with fingerprinting experiments, RNA/RNA hybridization studies generally showed a similar pattern of segment variability with an exception being segment L3 (Squire et al., 1986a; Kowalik and Li, 1987). These studies demonstrated that L3 was well conserved among BTV-10, 11, 13, and 17. They also demonstrated that BTV-13 was more closely related to the other serotypes than predicted by the RNA fingerprinting results. Kowalik and Li (1987) were the first to include the United States isolate of BTV-2 in their genetic relatedness comparisons and found that BTV-2 was related to BTV-10, 11, and 17. This study also verified that each set of segments across the serotypes (e.g., all of the L1 segments) were cognate genes. Genes that are termed cognate are those which encode proteins of similar function and can be inferred as such by hybridization studies (Gaillard and Joklik, 1982). By using a combination of differing hybridization and washing stringencies, Kowalik and Li (1987) also observed some variability among the S4 segments. Similar results have been seen among South African BTV-1, BTV-2 and 3 from Cyprus, and United States BTV-10 (Mertens et al., 1987b). No hybridization of BTV RNA with genomic RNA from viruses of different serogroups such as epizootic hemorrhagic disease virus (EHDV; Brown et al., 1988; Wilson et al., 1990), reovirus, bovine rotavirus, Nebraska calf disease

virus, the dsRNA from the virus-like particle of *Endothia parasitica* (Kowalik and Li, 1987), and bacteriophage Ø6 (Li *et al.*, 1987) were observed. This suggests that although several of these groups appear to be very similar, especially BTV and EHDV, they are only distantly related.

Hybridization studies of viral RNA with cDNA derived from BTV genome segments have also recently been done (Squire *et al.*, 1986a; 1986b; Huismans and Cloete, 1987; Unger *et al.*, 1988; Dunn and Stott, 1989; Dunn *et al.*, 1989; de Mattos *et al.*, 1989). These results show that segment L2 is serotype-specific even when this segment was individually hybridized with all 24 serotypes and hundreds of field isolates. They also demonstrated variability with segments M3, S1 and S4 and no differences in the hybridization intensities of the L3 segments as has been shown by the RNA/RNA hybridization studies. One major discrepancy is apparent between the RNA/RNA hybridization data and a cDNA/RNA hybridization study. Segment S2, which encodes NS2, was shown by direct RNA/RNA hybridization to be highly conserved among the United States BTV serotypes (Squire *et al.*, 1986a; Kowalik and Li, 1987). However, Unger *et al.* (1988), using what they believed to be a full clone of segment S2 from BTV-17, found that their cDNA probe only hybridized weakly with BTV-11 and not at all to BTV-2 and 13. The reason for this discrepancy is not clear. However, in this study and several of the others involving BTV clones, the researchers claimed to have

full-length clones of BTV segments but none of these groups have yet provided nucleotide sequence data to substantiate their assertions. Since, until this present study, it was very difficult to clone entire BTV genes, it should be assumed that these clones are not full-length. Therefore, caution should be exercised when interpreting the cDNA/RNA hybridization data. Again, there was no hybridization of BTV cDNA sequences with any other members of Reoviridae (Huismans and Cloete, 1987; Dunn et al., 1989; Unger et al., 1988; Wilson et al., 1990).

Indirect measures of viral relatedness of the United States serotypes were made using peptide and antigenic epitope mapping (Mecham et al., 1986; Li et al., 1989; Yang et al., 1990). Peptide mapping by several different digestion strategies showed VP2 (segment L2) and VP5 (segment M3) to be highly variable, VP6 (segment S3) and VP7 (segment S1) to be slightly variable, and the rest of the proteins to be well conserved. Each of the monoclonal antibodies directed against VP7 recognized an epitope in each of the five serotypes. The mapping of VP5 with one monoclonal antibody showed a common epitope among BTV-2, 10, 11, and 17 that was not shared with BTV-13. Interestingly, one monoclonal antibody directed against VP5 of BTV-10 recognized an epitope on the sigma 3 protein of reovirus serotype 2 and 3 but not the more distantly related serotype 1 (Yang et al., 1990). Alignments of nucleotide and deduced amino acid sequences of BTV-10 M3

and reovirus 3 S4, which encodes sigma 3, failed to identify any regions conserved between BTV and reovirus (Li and Kowalik, unpublished observations).

The best approach to determine the relationship among BTV serotypes is to directly analyze the nucleotide and amino acid sequences of the viruses. As the cloning and/or sequencing of the entire genome of each serotype or even of one dsRNA segment from several BTV isolates was unreasonable at the time, the relatedness of BTV serotypes was initially compared by terminal RNA sequencing (Rao et al., 1983b; Mertens and Sangar, 1985). All of the BTV termini examined, as well as those from Ibaraki virus (Mertens and Sangar, 1985) and EHDV-1 (Wilson, personal communication), contained 5'-GUUAAA-3' as the 5' terminus of the plus strand and 5'-ACUUAC$_{OH}$-3' at the 3' terminus. Reoviruses also retain terminal consensus sequences, although their nucleotide sequences are different from BTV (Li et al., 1980a; 1980b; Gaillard et al., 1982).

The nucleotide sequences of segment L2 from BTV-2, 10, 11, 13, and 17 have recently been completed (Fukusho et al., 1987; Ghiasi et al., 1987; Ritter and Roy, 1988; Yamaguchi et al., 1988a; 1988b). The segments are very variable with 50% to 70% of the nucleotides conserved. Surprisingly, there are unmatched nucleotides (from 3 to 77 unmatched nucleotides) within the VP2 open reading frame. These result in several shifts in the reading frames. VP2 is also quite variable with only 32% to 73% of the amino

acids conserved. This large degree of variability is most likely due to strong selective pressure exerted against VP2, as it is the virus neutralization antigen (Huismans and Erasmus, 1981). The pattern of relatedness among the five serotypes based on L2 sequence alignments is the same as that predicted by Kowalik and Li (1987); BTV-13 is the most distantly related of the United States serotypes, BTV-10, 11, and 17 are very closely related, and BTV-17 could have evolved from BTV-11 as predicted by Rao et al. (1983a). Further analyses of these and other BTV sequences will be necessary to further clarify the relationships among the United States BTV serotypes, especially the relationships among the three most closely related serotypes (BTV-10, 11, and 17).

JUSTIFICATION

Genetic data suggest that the United States prototypes BTV-10, 11, and 17 are closely related, with BTV-17 evolving from BTV-11. BTV-13 is much more distantly related to these viruses. The RNA/RNA hybridization data have shown that BTV-2 appears to be more closely related to BTV-10, 11, and 17 than BTV-13. However, due to the isolation of BTV-2 from the other serotypes and the proximity of its Florida location to the Caribbean, it has been postulated that the United States isolate of BTV-2 is derived from an unidentified Caribbean strain. Confirmation of these hypotheses will require sequence

analyses of entire BTV genomes or comparison of a marker region within the genome that would be representative of genetic flow in BTV. The United States prototype BTV serotypes would serve as a good model for a study of evolutionary relationships since the United States is essentially a "closed system" with regards to BTV infiltration and an accurate historical record of the appearance of new serotypes has been kept (Barber, 1979). This isolation is due to the United States being surrounded by water and the absence of BTV in Canada and Mexico. Only two documented external introductions of the BTV serogroup (BTV-10 in 1953 and BTV-2 in 1982) have been noted.

A worldwide distribution of 24 BTV serotypes would imply great diversity of this group or at least in the proteins and the genes from which the BTV serotypes are defined. Interestingly, nucleic acid hybridization data of the U.S. BTV serotypes as well as all 24 BTV serotypes indicate divergence primarily in the genes coding for VP2 (dsRNA segment L2) and VP5 (segment M3) and to a lesser extent VP7 (segment S1) and NS3/NS3a (segment S4). The other genomic segments (L1, L3, M1, M2, S2, and S3) are much more conserved.

Members of BTV are defined serologically, thus a potential BTV isolate is defined to the serogroup by a precipitin reaction with anti-BTV serogroup serum and assigned to a serotype by a neutralization reaction with an anti-BTV serotype serum. New serotypes are based upon

negative responses (i.e., no neutralization) with all serotype-specific sera. Therefore, in order for a virus isolate to be identified as BTV, the isolate must possess the BTV serogroup-specific antigen. Potentially, any recombinant virus, be it composed of dsRNA, ssRNA or more unlikely, a DNA genome containing the BTV serogroup antigen, would be defined as a bluetongue virus. An event of this sort, however unlikely, could possibly occur in coinfections with other members of Reoviridae and may provide a mechanism for BTV diversification. Reassortment/recombination may be an explanation for the weak cross-reactivity of some BTV serogroup-specific antisera with EHDV group-specific proteins.

The gene coding for VP7 (S1) is the best choice for the marker sequence described above since (1) it is required for a virus to be identified as BTV (serogroup determinant), (2) it is variable among the serotypes, and (3) VP7 is not under any known immunoselective pressure. Therefore, the comparison of S1 nucleic acid and deduced amino acid sequences will provide a measure of genetic flow in BTV. This will result in the production of the first phylogeny based on temporal and/or genetic distances for an orbivirus. The sequence data should also provide insight into the function(s) of VP7.

OBJECTIVES

The overall objectives of this project are to

characterize the relationship of the five United States prototype bluetongue viruses on a finer level than previously analyzed and use the resulting information to search for common features in this viral system in order to propose a relationship among the United States BTV serotypes as well as with other virus groups.

These objectives will be met as follows:

1. Develop better and less time-consuming techniques to grow and purify BTV in the quantities required for this project.
2. Develop methods to clone as well as directly sequence BTV genes in particular, and dsRNA in general.
3. Determine the nucleic acid and deduced amino acid sequences of segment S1 of BTV-2, 11, 13, and 17, and compare these results amongst each other and with segment S1 from BTV 10 (Yu et al., 1988).
4. Apply the sequence data obtained in (3) to produce a phylogenetic tree for these isolates and compare the results with predicted relationships based on the less direct measures described in the introduction.
5. Identify predicted structural features of the RNA and proteins that are common among the different serotypes.
6. Use any similar sequential or structural features found in segment S1 or VP7 to identify features found in common with other viral systems. Common features of BTV with other viruses will have significance in

evolutionary terms.

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CHAPTER II

SEQUENCE ANALYSES AND STRUCTURAL PREDICTIONS OF DOUBLE-STRANDED RNA SEGMENT S1 AND VP7 FROM UNITED STATES PROTOTYPE BLUETONGUE VIRUS SEROTYPES 13 AND 10

ABSTRACT

The nucleotide sequence of segment S1 and the deduced amino acid sequence of VP7 from bluetongue virus (BTV) serotype 13 was determined. Sequences were obtained by use of standard dideoxy DNA sequencing and by direct sequencing of genomic double-stranded RNA (dsRNA). The dsRNA was sequenced with a new dideoxy protocol that produces 300 to 350 bases per set of reactions. Segment S1 is 1156 bp long and contains one long open reading frame capable of coding for 349 amino acids. The protein, VP7, has a calculated molecular weight of 38,619 and a net charge of +1.5 at pH 7.0. Segment S1 of BTV-13 has 79.6% of its nucleotides conserved when compared with segment S1 of BTV-10. While most of these differences occur at the third codon position of the open reading frame, the differences between the 89 base long, 3' noncoding regions occur predominantly in pockets at positions 1092-1098, 1112-1114 and 1125-1129. Potential stem-loop structures encompassing the stop codon of the open reading frame are proposed for both serotypes.

Comparisons of VP7 from BTV-13 and BTV-10 indicate that 93.7% of the amino acid residues are conserved, including a single lysine at position 255. Secondary structure predictions infer an eight-stranded ß-barrel structure between residues 150 and 250. This putative ß-barrel may serve as a target for the development of drugs to combat bluetongue disease. Comparable structures detected in the core proteins of single-stranded RNA viruses from both plants and animals suggest that these viruses and BTV had a common origin.

INTRODUCTION

Bluetongue virus (BTV) is the prototype of the genus Orbivirus in the family Reoviridae. These arthropod-borne viruses have similar morphological and physiological features (see Gorman et al., 1983).

There are 24 serotypes of BTV in the world, 5 of which (BTV-2, 10, 11, 13 and 17) have been isolated in the United States. The genetic relationships among these five serotypes have been investigated by oligonucleotide fingerprinting (Sugiyama et al., 1981; 1982; Rao et al., 1983), RNA/RNA hybridization (Squire et al., 1986; Kowalik and Li, 1987), and by hybridization of cloned DNA with genomic RNA (Huismans and Cloete, 1987). Sequence analysis is necessary to more fully understand the evolutionary relationships among BTV serotypes.

Of the 10 genomic dsRNA segments of BTV, segment S1 is

the most representative of the BTV genome since it encodes the group-specific antigen, VP7, which is also the major core protein of BTV (Huismans and Erasmus, 1981). Sequence analysis and structure/function predictions of VP7 will be useful in understanding the relationships of BTV with other viruses. This information will also be applicable in the future development of diagnostic tests for BTV infections and in the development of pharmaceuticals for treatment of bluetongue disease.

We present an improved virus isolation protocol that results in the rapid purification of all five U.S. BTV serotypes, a new dsRNA sequencing procedure using reverse transcriptase and dideoxy nucleotides, and the nucleotide sequence and the deduced amino acid sequence of dsRNA segment S1 and VP7 from BTV-13. These data are compared with their complements from BTV-10 (Yu et al., 1988). Potential secondary structures at the 3' terminus of the plus strand from both serotypes are proposed. Structural predictions and comparisons of VP7 from BTV with core proteins of icosahedral, ssRNA viruses of both plants and animals suggest that these groups evolved from a common precursor. It is proposed that this structural data be applied toward the development of antiviral agents to be used in the treatment of BTV infections as has been done with some members of Picornaviridae.

MATERIALS AND METHODS

Virus and cell culture

United States prototype BTV-13 was obtained from the Arthropod-Borne Animal Diseases Research Laboratory (Laramie, Wyoming) and plaque-purified three times in mouse L cells.

Baby hamster kidney cells (BHK) were used to produce BTV and mouse L cells were used to titer the virus stocks. The BHK and L cell monolayers were maintained in Eagle's minimum essential medium supplemented with 5% calf serum (Hyclone Laboratories) at 37° in a humidified 5% CO_2 atmosphere in the absence of antibiotics.

BHK cells were infected with BTV at a multiplicity of infection of 1 plaque forming unit. Infected cells were harvested for virus purification 3 days post infection. Seed stock virus was prepared in BHK cells and titered by plaque assay in L cells as described in Howell et al. (1967), except that Sephadex G-75 was used as an overlay instead of agarose.

Purification of virus and genomic dsRNA

Virus particles were purified from infected BHK cells by combining and modifying procedures developed elsewhere (Mertens et al., 1984; 1987). Infected cells were scraped from roller bottles, pelleted at 10,000 x g for 10 min and resuspended in NTE (200 mM Tris, pH 8.0, 100 mM NaCl, 2 mM

EDTA) containing 1% (w/v) Triton X-100 and 0.5% (w/v) deoxycholate. The cells were incubated for 10 min at room temperature. Particulates were removed by centrifugation at 2,000 x g for 5 min at room temperature. The supernatant was then loaded onto a 40% (w/v)/66% (w/w) sucrose step gradient containing 200 mM Tris, pH 8.0, and centrifuged in a Beckman SW41Ti rotor at 39,000 rpm for 1.5 hr at 4°. A band at the interface of the two sucrose solutions containing partially-purified virus was collected and resuspended in 200 mM Tris, pH 8.0, containing 1% (w/v) n-lauryl sarcosine and 10 mM dithiothreitol (DTT). The partially purified virus was incubated at 37° for 30 min or at 4° overnight to resuspend the precipitate. Virus particles were then purified to homogeneity and concentrated by pelleting them through 40% (w/v) sucrose in 200 mM Tris (pH 8.0) by centrifugation in a Beckman SW41Ti rotor using the same conditions described above. The purified virus was resuspended in 200 mM Tris, pH 8.0, and stored at 4°.

Genomic dsRNA was released from purified virions by incubating the virus at 55° for 30 min in the presence of 1% (w/v) SDS. Viral proteins were removed by phenol extraction and the dsRNA was concentrated by ethanol precipitation.

Cloning of genomic dsRNA

DNA clones of BTV genes were made according to Huismans

and Cloete (1987) except that 20 mM methylmercuryhydroxide (MeHgOH) was consistently used to denature the dsRNA in the cloning reactions. A DNA clone corresponding to segment S1 of BTV-13 (11.21-J) was identified when it hybridized only to genomic dsRNA segment S1 of BTV-13 when reacted with BTV-13 genomic dsRNA which had been separated by polyacrylamide gel electrophoresis and alkaline blotted to positively-charged membranes (Kowalik and Li, 1987; Li *et al.*, 1987).

cDNA sequencing

Clone 11.21-J, representing the 3' half of the coding strand of dsRNA segment S1, was sequenced in both directions using Klenow fragment (Promega) and Sequenase (US Biochemicals) in dideoxy sequencing reactions (Sanger *et al.*, 1977) in accordance with the manufacturer's instructions.

Direct sequencing of dsRNA using separate primer extension and termination reactions

Both strands of the 5' half of BTV-13 dsRNA segment S1 were directly sequenced using reverse transcriptase in a new RNA sequencing protocol employing separate extension and termination reactions. Total dsRNA (3 to 6 μg) was denatured by the addition of MeHgOH to a final concentration of 20 mM and incubated at room temperature for 15 min. Sodium acetate (pH 4.8) was added to a concentration of 250 mM and the denatured RNA was

precipitated by the addition of 2.5 vol of ethanol and incubated at -70° for 5 min. The RNA was then pelleted by centrifugation in a microfuge, dried and resuspended in 4 µl of water. Reagents for the sequencing reaction were added in the following order: 2.5 ng of primer, 4 µl of 5X RT buffer (1X RT buffer is 50 mM Tris, pH 8.3, 50 mM MgCl, 10 mM KCl, 10 mM DTT), 4 µl of actinomycin D (400 µg/ml; Boehringer Mannheim Biochemicals), 2 µl of extension mix (25 µM each of dCTP, dGTP and TTP), 25 µCi of ^{32}P-dATP (800 Ci/mmol; Amersham), 20 U of RNasin (Promega), and 25 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) in a final volume of 20 µl. The reaction mixture was incubated at 42° for 10 min. Extension was terminated by transferring 4.5 µl aliquots of this solution to each of four tubes containing 2.5 µl of the specified termination mixes (Table 1) and incubated at 42° for 10 min. In order to sequence the termini of the dsRNA, 5 units of terminal deoxynucleotidyl transferase (Boehringer Mannheim Biochemicals) was added to the termination reaction and the mixes incubated for an additional 5 min. Reactions were stopped by the addition of 5 µl of formamide that contained bromophenol blue and xylene cyanol as marker dyes.

DNA and RNA sequencing reaction products were resolved by electrophoresis through 6% polyacrylamide gels containing 8 M urea and 1.5 X TBE (1X TBE is 90 mM Tris, pH 8.0, 90 mM boric acid, 2 mM EDTA). After electrophoresis,

TABLE 1

Concentration (µM) of Nucleotides in Termination Mixes.

	Termination Mix			
	A	C	G	T
dATP	25	25	25	25
dCTP	250	25	250	250
dGTP	250	250	25	250
dTTP	250	250	250	25
ddATP	12.5			
ddCTP		50		
ddGTP			50	
ddTTP				100

the gel was transferred to 3MM paper (Whatman), covered in plastic wrap and exposed to x-ray film at -70° without intensifying screens.

RESULTS

Sequencing BTV dsRNA

Protocols previously devised to sequence dsRNA using reverse transcriptase and standard dideoxy reactions (Bassel-Duby et al., 1986; Weiner and Joklik, 1987) did not yield readable sequences when BTV dsRNA was the template (unpublished observations). For this reason, we developed the procedure described under Materials and Methods. This approach was based on the protocol of Tabor and Richardson (1987) in which separate extension and termination reactions were used to sequence DNA with a chemically-modified T7 DNA polymerase (Sequenase; U.S. Biochemical). The separate extension and termination steps developed for dsRNA facilitate the optimization of the sequencing reaction for a particular RNA species. Under these conditions, 300 to greater than 350 bases of sequence data were acquired per set of reactions.

Nucleotide Sequence Analysis of Segment S1

The nucleotide sequence of segment S1 of BTV-13 has been determined by a combination of direct RNA sequencing and the sequencing of a partial DNA clone (Fig. 1). This dsRNA segment is 1156 bp long with a single long open

Figure 1. Nucleotide sequence of the plus strand of segment S1 from BTV-13. The start and stop codons are in bold print. Mismatches found in segment S1 of BTV-10 (Yu *et al.*, 1988) are shown for comparison. Variable pockets in the 3' noncoding region are identified with boxes.

```
GUUAAAAAUCUCUAGAG AUG GAC ACU AUC GCA GCA AGA GCU CUC ACU GUG AUG CGG GUA
           A                        C           A                   A  C

UGU GCU ACC UUA CAA GAG GCC CGA AUU GUC CUA GAA CCC AAU GUA AUG GAG AUA CUG GGA
          G C U           A A         G U G     G         G       A   U U     G

AUA GCA AUC AAU AGA UAU AAU GGA UUA ACU CUA CGU GGA GUU ACG AUG AGA CCC ACU UCA
      U           G   C         C C     T     A       G         C C   G   C   G

UUA GCU CAA AGA AAU GAA AUG UUU UUC AUG UGU UUA GAU AUG AUG GUG UCA GCA GCG GGA
      A               G           U           G             C     U   U   U   U

AUA AAU GUC GGA CCA AUC UCA CCC GAU UAU ACG CAA CAU AUG GCU ACU AUA GGU GUG CUG
          U       G   A   G   A   C       U                   G   U       A   A

GCA ACG CCU GAG AUA CCG UUC ACA ACU GAG GCA GCG AAU GAG AUU GCU CGU GUU ACA GGA
      A   G   A       U   U       G   A   G           A   A       A   G   U   G

GAG ACU UCA ACA UGG GGA CCG GCG CGU CAG CCU UAC GGA UUU UUC CUU GAA ACU GAA GAG
          G           G   A                   U   U   C                   G   A

GUU UAU CAA CCU GGA AGA UGG UUC AUG CGA GCU GCU CAA GUU GUU ACA CCA GUG GUU UGU
ACC  UC       A   G   G               C   C          CA   A   U G     A   G   C

GGA CCA AAU AUG GUU CAG GUU UCA CUU AAU GCU GGA GCG AUA GGU GAU GUG CAG CAG AUC
  U   G G       A     A   G                          G    A       A   A       A

UUU CAA GGU CGU AAU GAU CCC AUG AUG AUU UAU UUA GUU UGG AGA CGG AUC GAG AAC UUC
      G                               A           G       G A A       A       U

UCC AUG CCG CAG GGU AAU UCA CAG CGC ACG UUA GCU GGC GUC ACU GUG AGU GUG GGU GGU
G G     G     A                  AA   U CA    G   U   G       C       U       A

GUG GAU AUG AGA GCG GGG CGC AUA AUC GCA UGG GAU GGG CAG GCG GUG CUA CAA AUU CAC
  U   C       G       A       U   A   G           A       C  C    G   U G G   U

AAU CCG ACU CAA CAA AAU GCU AUG GUA CAA AUU CAG GUG GUG UUU UAC GUU UCC AUG GAU
          A       G       G       G       A       U       C   U A A   U

AAG ACC CUC AAC CAG UAC CCC GCA CUA ACG GCA GAA AUA UUU AAU GUG UAC AGC UUU AGA
  A   U U A                   U U G   U   U   G   U   C       U           C   G

GAC CAC ACU UGG CAU GGA CUG AGG ACC GCU AUA CUA AAC AGA ACA ACG CUU CCA AAU AUG
          A           G   A   A   G   G     U             C   A   G       C

UUA CCU CCA AUC UUU CCG CCG AAU GAC CGG GAC AGU GUA CUC ACG AUU CUG CUA CUG UCG
C G   A           C   A   A       U   A   U   C A C U A   U C     A   U U A   U

ACG CUC GCA GAU GUU UAU UCA GUU UUG AGA CCU GAG UUU GCA AUC CAC GGC GUA AAU CCA
  A   U   U           C A U       A   G   A           G   U                   G

AUG CCU GGC CCG CUU ACA CGU GCG AUU GCU CGC GCC GCU UAC GCA UAGUCCACUUUGCACGGGU
      A   G       C           U       G           C   U  UG

GUGGGUUA|UGCGGGU|GGUGUGUCGGUUG|CAA|GAAAUAUGUA|UCUUG|UUUAAACGUCUCUGGGAUUACACUUAC
        |CAUAU C|             |UGG|          |A CCA|             UA
```

reading frame of 349 codons starting at position 18 and ending at position 1064. Comparisons with the nucleotide sequence of the cognate gene of BTV-10 (Yu et al., 1988) show that the lengths of the entire segment, open reading frame and noncoding region are all conserved (Fig. 1). The two S1 segments are identical at 79.6% of the nucleotide positions and have very similar G/C contents (46.7% for BTV-13 and 46.8% for BTV-10). The 5' and 3' termini of the coding strand are also well conserved with 96.7% (29 of the first 30 residues) and 92.6% (25 of the last 27 residues) of the respective termini of the two segments matched. It has previously been noted that there is a long 3' untranslated region in segment S1 of BTV (Yu et al., 1988). Within this 89 base stretch, 13 of the 15 nucleotide differences (87%) are contained in three pockets at positions 1092-1098, 1112-1114 and 1125-1129 (Fig. 1). Therefore, most of the variability between the two S1 segments occurs in the coding region.

Amino acid sequence analysis of VP7

The deduced amino acid sequences of VP7 from BTV-13 and BTV-10 are compared in Fig. 2. Both proteins are composed of 349 amino acids and 93.7% of the residues are either matched or represent conservative changes. The calculated molecular weight of VP7 from BTV-13 is 38,619 with 26 acidic and 25 basic residues, and 5 histidines resulting in an estimated net charge of +1.5 at pH 7.0. As compared

Figure 2. Deduced amino acid sequence of VP7 from BTV-13. Mismatches in the amino acid sequence of VP7 from BTV-10 (Yu et al., 1988) are shown below the BTV-13 sequence. Plain text identifies conservative substitutions and bold print shows unique differences. The single conserved lysine residue at position 255 is boxed.

```
M D T I A A R A L T   V M R V C A T L Q E   A R I V L E P N V M   E I L G I A I N R Y
                            A                           A

N G L T L R G V T M   R P T S L A Q R N E   M F F M C L D M M V   S A A G I N V G P I
                                                              L

S P D Y T Q H M A T   I G V L A T P E I P   F T T E A A N E I A   R V T G E T S T W G

P A R Q P Y G F F L   E T E E V Y Q P G R   W F M R A A Q V V T   P V V C G P N M V Q
                              T F                         A       A           D   I

V S L N A G A I G D   V Q Q I F Q G R N D   P M M I Y L V W R R   I E N F S M P Q G N
              R                                                           A   A

S Q R T L A G V T V   S V G G V D M R A G   R I I A W D G Q A V   L Q I H N P T Q Q N
    Q   Q                                                     A     H V

A M V Q I Q V V F Y   V S M D K T L N Q Y   P A L T A E I F N V   Y S F R D H T W H G
                      I

L R T A I L N R T T   L P N M L P P I F P   P N D R D S V L T I   L L L S T L A D V Y
                                                        I     L

S V L R P E F A I H   G V N P M P G P L T   R A I A R A A Y A
                                                            V
```

TABLE 2

Deduced Amino Acid Composition of VP7

Amino acid	BTV-13 VP7 residue #	BTV-13 VP7 residue %	BTV-10 VP7 residue #	BTV-10 VP7 residue %
A	34	9.7	40	11.4
R	24	6.9	24	6.9
D	19	5.4	18	5.1
N	12	3.4	13	3.7
C	3	0.9	3	0.9
E	19	5.4	20	5.7
Q	14	4.0	14	4.0
G	23	6.6	23	6.6
H	5	1.4	6	1.7
I	24	6.9	24	6.9
L	27	7.7	28	8.0
K	1	0.3	1	0.3
M	19	5.4	19	5.4
F	13	3.7	14	4.0
P	24	6.9	21	6.0
S	13	3.7	11	3.1
T	28	8.0	30	8.6
W	5	1.4	5	1.4
Y	10	2.9	9	2.6
V	32	9.2	29	7.4
Total	**349**	**100**	**349**	**100**
Charge	**+1.5**			**+1**
Size	**38,619**			**38,551**

with BTV-13, VP7 from BTV-10 has similar residue frequencies, a MW of 38,551 and a net charge of +1. Both proteins contain many hydrophobic amino acids but have only a single lysine residue, which is conserved at position 255 (Table 2).

DISCUSSION

BTV-13 has been grown in BHK cells and purified to homogeneity by modification of a published protocol (Mertens et al., 1987). This procedure reduces the time required to purify and concentrate BTV from approximately 16 hr to less than 5 hr. Both approaches result in purified virus from all five U.S. BTV serotypes (unpublished observations). In addition, the new protocol includes NS3 and NS3a in the purified virion (unpublished observations). Thus these two proteins, which are encoded by segment S4, may actually be structural proteins, not nonstructural proteins as previously described (Mertens et al., 1984; Van Dijk and Huismans, 1988).

Attempts to sequence BTV dsRNA segments with two procedures developed for reovirus (Bassel-Duby et al., 1986; Wiener and Joklik, 1987) resulted in poor or unreadable sequence data, even though both worked well with reovirus in control experiments (unpublished observations). Therefore, we developed an alternate approach based on separate extension and termination reactions which produces clean, readable sequences of BTV and reovirus dsRNA

segments as well as ssRNA templates (data not shown).

The nucleotide and deduced amino acid sequences of the gene (segment S1) coding for the major core protein and group antigen (VP7) of BTV-13 have been determined by direct RNA and cDNA sequencing. The dsRNA segment is 1156 bp in length with only a single long open reading frame of 1047 bases and can code for a protein of 349 amino acids. The calculated molecular weights of VP7 from BTV-13 and BTV-10 (38,619 Da and 38,551 Da, respectively) are less than estimates made from SDS-PAGE (approximately 42,000 Da each). The reason for this discrepancy is not known.

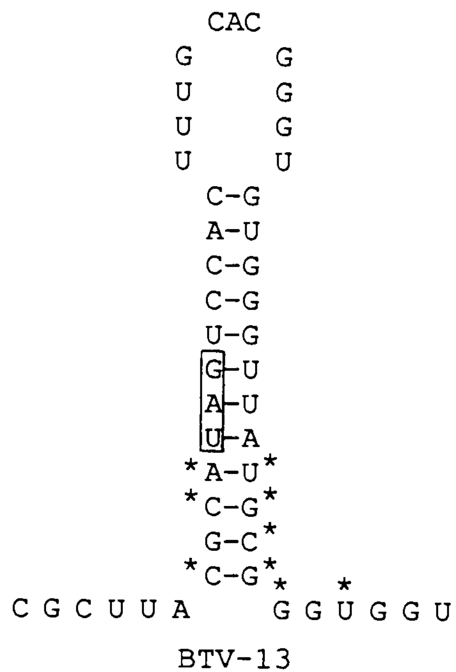
The VP7 proteins are better conserved than the dsRNA because the majority of the nucleotide differences (83%) within the long open reading frame are at the third codon position and usually do not alter the amino acid sequence. Only 12.4% of the nucleotide changes occur at position 1 and 4.6% of these differences are found at position 2. The protein sequences of VP7 from BTV-13 and BTV-10 show a high degree of conservation with 93.7% of the residue positions representing either identical or conservative amino acid substitutions. The biological significance of the single lysine residue in both VP7 proteins and its conservation at position 255 is not known. However, by specifically cleaving VP7 at this residue with endoproteinase Lys-C (Boehringer Mannheim Biochemicals), an antigenic epitope conserved in both BTV-13 and BTV-10 has been mapped to the larger, N-terminal VP7 peptide of each serotype using a

monoclonal antibody made against VP7 of BTV-10 (data not shown).

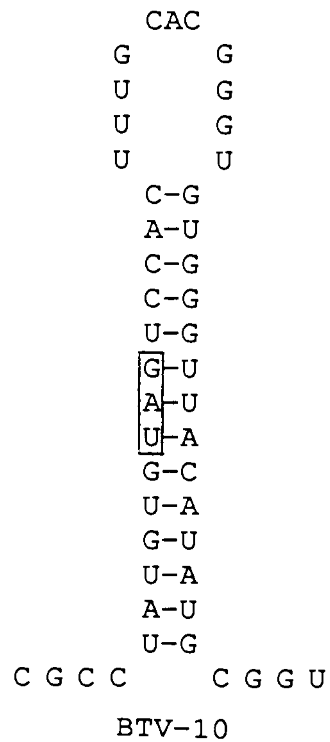
The pattern of conservation of the dsRNA termini of the S1 segments differs from that observed in the open reading frame. The long, 3' noncoding termini of the S1 segments do not appear to have accumulated random changes in the nucleotide sequence. Most of the differences are limited to a few locations (Fig. 1). Interestingly, these 3' termini can potentially form stable secondary structures that include the termination codon of each open reading frame (Fig. 3). The results are stem-loop structures with a ΔG = -18.9 kcal/mol for BTV-13 and a ΔG = -14.6 kcal/mol for BTV-10. The loops are each 11 bases long while the stem of BTV-13 contains 12 base pairs, two less than in BTV-10. One of the pockets of variability in the noncoding region occurs at the base of the stem structure. The first base change in this pocket (U>C) increases the stability of the beginning of the stem structure. Two of the differences observed in the next 3 G-C base pairs reduce the overall stem stability. However, the stem is elongated by two additional base pairs since the G residue at position 1096 in segment S1 of BTV-13 is a U in BTV-10, thus permitting an A-U match followed by a U-G. These differences result in a stem loop in segment S1 of BTV-10 that is two base pairs longer and has a free energy which is lower by 4.3 kcal/mol. Therefore, the longer stem length of segment S1 of BTV-10 may be necessary to add

Figure 3. Potential secondary structures of the 3' termini of the plus strands of segments S1 of (A) BTV-13 and (B) BTV-10. Positions 1055 to 1101 of the nucleotide sequences from both serotypes are shown. The stop codon in each structure is boxed. Locations where nucleotides differ between the two serotypes are marked with asterisks in (A). Estimates of the loss of free energy (ΔG; Salser, 1977) due to the folding of each putative structure is provided below the respective diagram.

A



B

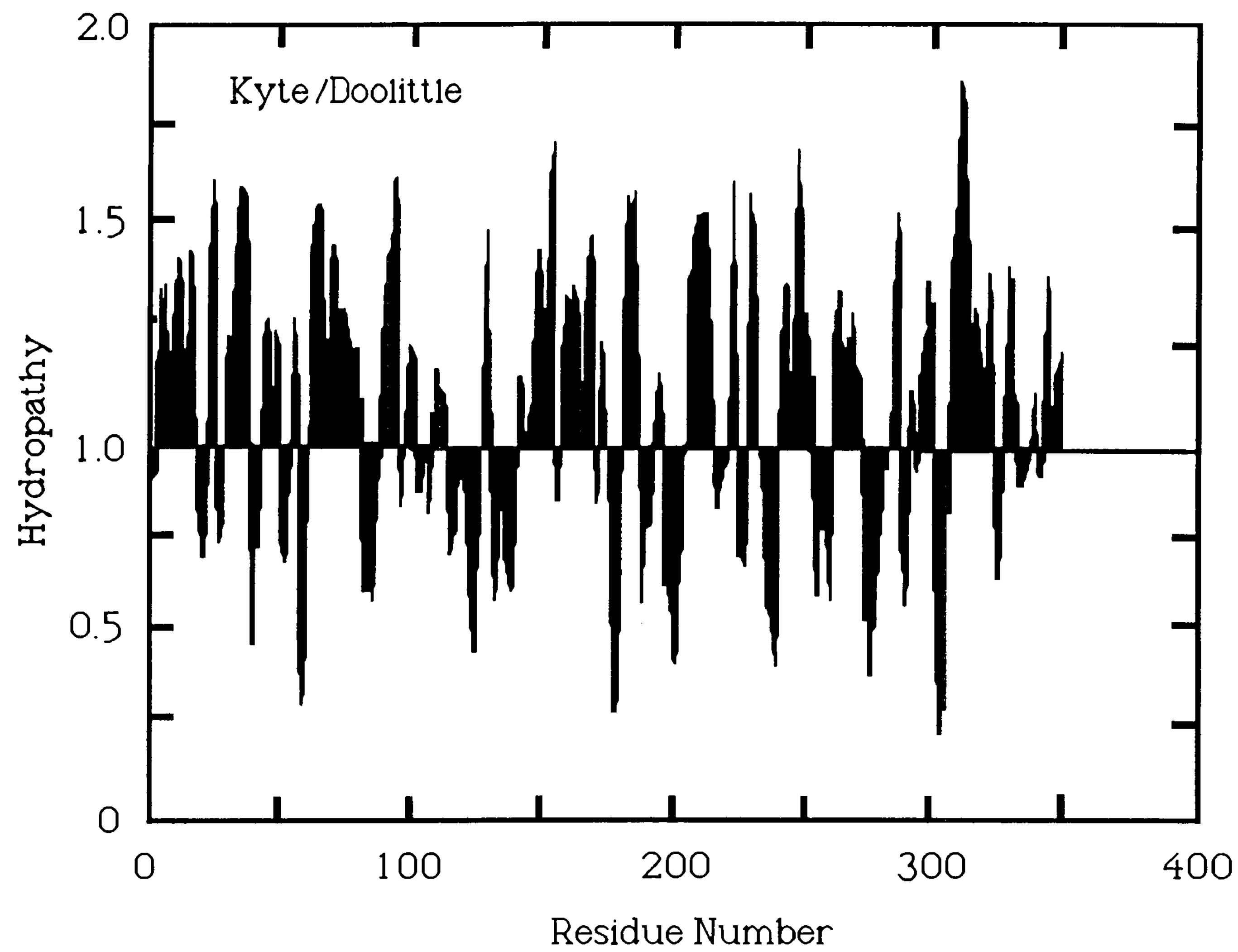


stability to its stem-loop structure. Segment S2 of South African prototype BTV-10 (Hall et al., 1989) appears to possess a stem-loop of similar structure, stability and relative location (incorporating the stop codon of the open reading frame; unpublished observations) as does segment S1 of reovirus serotype 2 (Li et al., 1980). The significance of these observations is not known, but these conserved structures may be necessary for some aspect of viral replication. Perhaps these stems and loops serve as recognition signals for the binding of a protein(s) such as NS2 from BTV, which has an affinity for BTV mRNA (Huismans et al., 1987). Using site-specific mutagenesis, Esteban et al. (1989) have recently shown that a predicted stem loop structure (-9 kcal/mol) in the 3' noncoding region of the L-A dsRNA virus of yeast is essential for replication of ssRNA template into dsRNA in an in vitro assay. Alternatively, the predicted structures may serve as attachment sites for some or all of the other BTV mRNA molecules, resulting in the proper selection of only one of each segment of RNA and serve as a nucleation point thus leading to the formation of a subviral particle with replicase activity. If accurate, this latter hypothesis could help answer one of the most perplexing questions in virology, namely: How does a dsRNA virus segregate and package one, and apparently only one, of each segment of genomic RNA into a mature, infectious virion during replication?

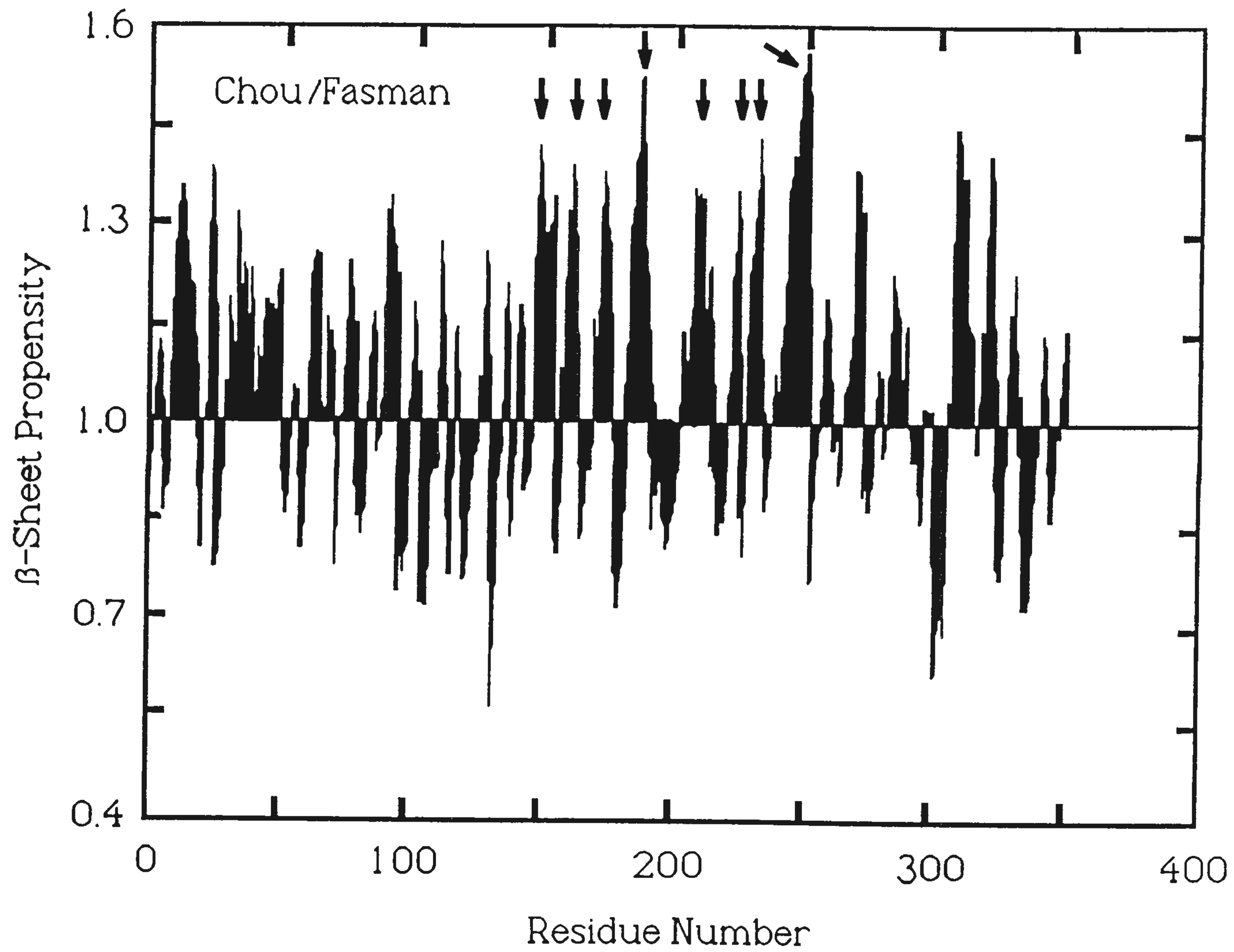
Potential secondary structure can be predicted from the conserved nature of VP7 from BTV-13 and BTV-10 (Fig. 4; BTV-10 data not shown). Both protein sequences fluctuate between very hydrophobic and hydrophilic stretches of amino acids (Fig. 4a). Chou/Fasman predictions (Chou and Fasman, 1974) show significant ß-sheet propensities between residues 150 and 250 (Fig. 4b). A high degree of ß-sheet structure is also predicted in this region when the sequences are examined by an algorithm based on data in Garnier et al. (1978; data not shown). Similar structures are suggested when human rhinovirus 14 (HRV14) VP1 and VP2 sequences (Callahan et al., 1985) are analyzed using the same critera (unpublished observations). These patterns are very reminiscent of ß-barrels. The three dimensional structures of several icosahedral RNA viruses from both plants and animals show that the major capsid structure of these viruses is an eight-stranded antiparallel ß-barrel (see Rossmann, 1988). An eight-stranded antiparallel ß-barrel has recently been predicted for the p24 core protein of HIV by secondary structure predictions and analogies with picornaviruses (Argos, 1989). A putative eight-stranded ß-barrel of VP7 is identified in Fig. 4b with the predicted ß-sheets of the ß-barrel marked. This structure also appears to have conserved the "puff" region between the fourth and fifth ß-sheet as has been observed in HRV14 VP2 and predicted in p24 of HIV (Rossmann et al., 1985, Argos, 1989). The conservation of these structures

Figure 4. Plots of hydropathy and ß-sheet propensity of VP7 from BTV-13. (A) Hydropathy plot of VP7 from BTV-13. Plot is based on Kyte and Doolittle (1982). Peaks above the meridian represent hydrophobic regions and projections below this line identify hydrophilic stretches. (B) Chou and Fasman protein structure prediction of VP7 from BTV-13 (Chou and Fasman, 1974). The ß-sheet prediction is shown. Residues represented in the larger peaks have a greater propensity to be included in a ß-sheet. An arrow denotes each of the proposed ß-sheets within the eight-stranded ß-barrel structure.

A



B



among viruses has been used in support of the hypothesis that the ssRNA viruses evolved from a common precursor. The data presented here suggest that at least BTV, and possibly all of the viruses in the Reoviridae family, may have evolved from this same common precursor.

Although speculative, the presence of a ß-barrel structure in the major core protein of BTV would be useful in developing a therapeutic regimen for bluetongue disease. Drugs have been produced that increase the rigidity of ß-barrel structures of the core proteins of picornaviruses, thereby inhibiting proper viral replication (Lonberg-Holm et al., 1975; McSharry et al., 1979; McKinlay et al., 1986). Rossmann (1988) has proposed that a similar rationale be used to create new drugs with analogous activities against other viruses (such as HIV; Argos, 1989), which contain a ß-barrel as the major structural motif of the viral capsid. The putative eight-stranded ß-barrel of VP7 should, therefore, prove to be useful as a target for the development of a similar therapy against bluetongue disease.

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CHAPTER III

CLONING AND AMPLIFICATION OF DOUBLE-STRANDED RNA

ABSTRACT

A method to clone and amplify double-stranded (ds) RNAs (ClampR) is described. This one-tube procedure combines cloning of denatured dsRNA using reverse transcriptase and the selective amplification of full-length DNA clones by the polymerase chain reaction. ClampR greatly reduces the amount of time and starting material required to clone and identify full-length DNA clones made from dsRNA. By using the conserved sequence of the 5' ends of bluetongue virus (BTV) genomic dsRNA segments, ClampR was used to clone and amplify all 10 dsRNA segments from the five BTV serotypes found in the United States. This amounts to nearly 100,000 base pairs (bp) of dsRNA cloned as unique full-length DNA copies. Clones of the genome segment (S1) encoding the bluetongue virus (BTV) serogroup antigen (VP7) were sequenced and shown to be a complete copy, containing 1156 bp and a single long open reading frame encoding a protein (VP7) of 349 amino acids. The utility of the ClampR reaction should make it widely applicable to rapid cloning and amplification of RNAs from many sources and greatly facilitate molecular studies.

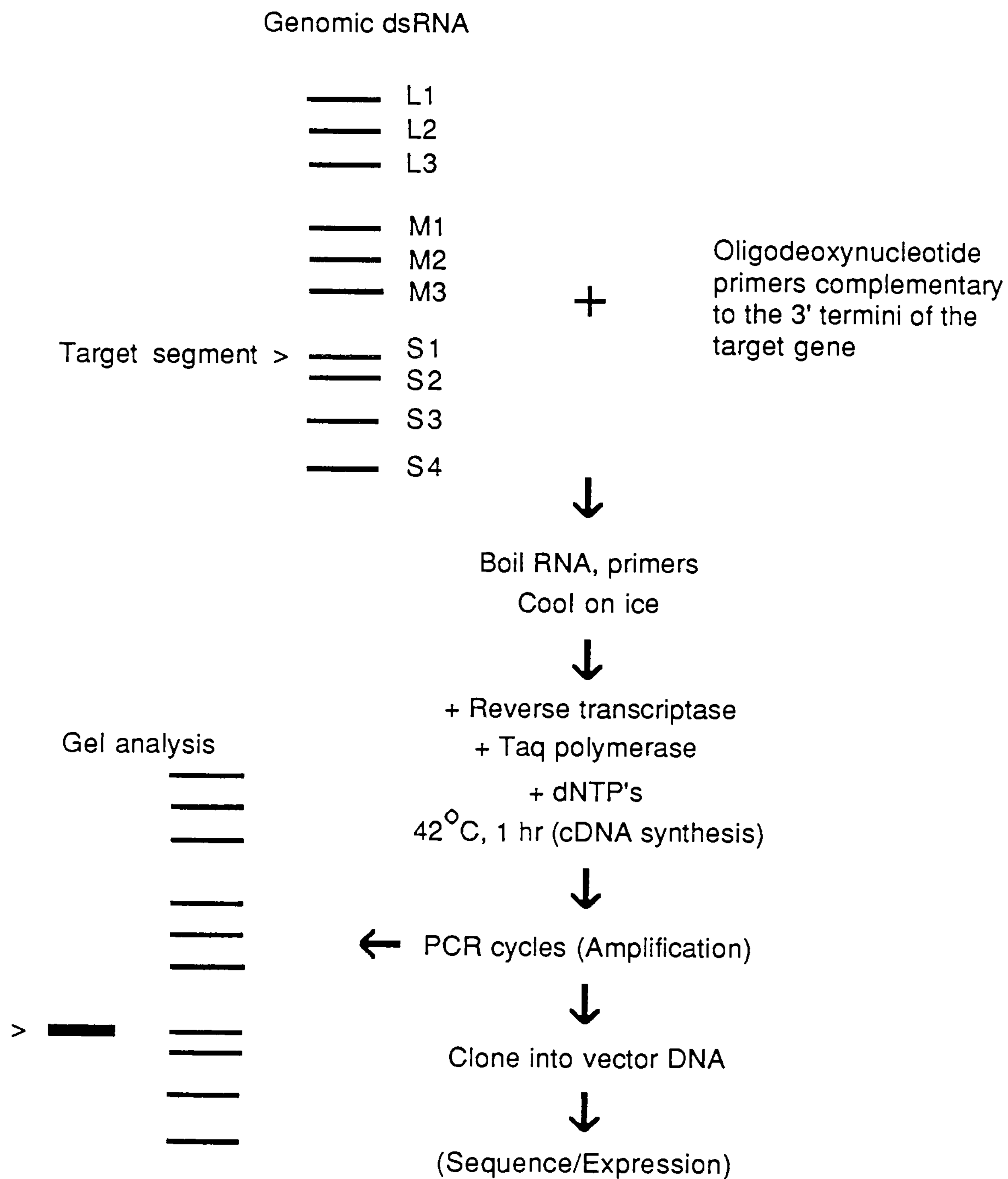
The Reoviridae family contains viruses with genomes

composed of segmented, double-stranded RNA (dsRNA). The dsRNA viruses are represented in all five taxonomic kingdoms with animal, plant and fungal dsRNA viruses being the most studied and of greatest economic importance (1). The *Orbivirus* genus of Reoviridae is comprised of arthropod-borne viruses of which bluetongue virus (BTV) is the prototype (2). BTV is spread by *Culicoides* spp. and is the causative agent of bluetongue, a degenerative and sometimes fatal disease of ruminants (3). At least 24 BTV serotypes exist worldwide, of which five (BTV-2, 10, 11, 13, and 17) are found in the United States. Because BTV is the model system for the *Orbivirus* genus and causes a debilitating condition in livestock, studies are underway to determine the molecular relationships among the BTV serotypes in the United States and abroad (4).

To obtain sufficient material for in-depth molecular studies of BTV, we coupled the polymerase chain reaction (PCR; 5) with a cloning step as part of a one-tube reaction to clone and amplify dsRNA (ClampR) into full-length DNA product. The ease and utility of this procedure is demonstrated by the cloning and amplification of full-length DNA for sequence analysis of segment S1 of BTV-11 and the production of complete copies of all 10 dsRNA segments of the 5 U.S. BTV serotypes (50 segments total).

The ClampR procedure is summarized in Fig. 1. Primers

Figure 1. General strategy for the cloning and amplification of specific dsRNA segments. Total genomic dsRNA is combined with a molar excess of primers containing a restriction endonuclease site and sequence complementary to the 3' ends of the dsRNA segment of interest. The dsRNA is denatured and the primer-RNA solution mixed with the four deoxyribonucleotide triphosphates, reverse transcriptase, and Taq polymerase. Primer annealing and copy DNA (cDNA) synthesis by reverse transcriptase occur at 42°C. The cloning portion generally results in the production of mostly small, incomplete copy DNA with full-length cDNA representing a minor component. A modified polymerase chain reaction using Taq polymerase is subsequently applied to preferentially amplify any complete cDNA produced in the first part of the ClampR reaction. Aliquots representing 10% to 20% of the total reaction are analyzed by agarose gel electrophoresis to ensure the production of full-length cDNA. ClampR reaction products are then digested with the primer-encoded restriction endonuclease, gel purified and ligated into an appropriate DNA vector for subsequent analyses.



Genomic dsRNA
L1
L2
L3
M1
M2
M3
Target segment >
S1
S2
S3
S4
+
Oligodeoxynucleotide primers complementary to the 3' termini of the target gene
Boil RNA, primers
Cool on ice
+ Reverse transcriptase
+ Taq polymerase
+ dNTP's
42°C, 1 hr (cDNA synthesis)
Gel analysis
PCR cycles (Amplification)
>
Clone into vector DNA
(Sequence/Expression)

specific for the termini of the target gene are annealed with denatured dsRNA, and cDNA synthesized by reverse transcriptase. Complete copy DNA is then selectively amplified to microgram quantities by PCR. Full-length clones are isolated by agarose gel electrophoresis and ligated into plasmid vectors for subsequent analyses.

The amplification reaction conditions were determined initially by using a DNA clone of segment S1 of reovirus type 3 (Dearing strain). PCR cycles were linked to a DNA cloning step to amplify cDNA from dsRNA (6). Primers used to amplify reovirus S1 DNA clone were mixed with total genomic dsRNA from reovirus type 3 and subjected to the ClampR procedure (Fig. 2, lane 4). Likewise, primers specific for the S1 segment of BTV-13 were used to clone and amplify S1 from total genomic dsRNA of BTV-13 (Fig. 2, lane 2).

The sensitivity of the ClampR procedure was tested and further optimized by cloning and amplifying the S1 segment of BTV (Fig. 3A) or segment S1 of reovirus (Fig. 3B) in the presence of decreasing amounts of starting dsRNA template. Even with less than 500 pg of total template dsRNA, a small aliquot (10 to 20%) of the ClampR reaction product was sufficient to demonstrate the presence of full-length DNA copies. Since the S1 segments of both viruses represent approximately 5% of the starting dsRNA, less than 25 pg of the input RNA can serve as template under these conditions.

Figure 2. Cloning and amplification of specific dsRNA segments. Total genomic dsRNA (500 ng) from reovirus type 3 or BTV-13 were mixed with primers (500 ng each) complementary to the 3' termini of the S1 segment of each virus. The nucleic acids were denatured by boiling, cooled on ice and added to the ClampR reaction mix. The cDNA was synthesized using reverse transcriptase, and amplified for 40 PCR cycles under the conditions described in (6). Aliquots (20%) of the reactions were analyzed by agarose gel electrophoresis. Lane 1: BstEII-digested lambda phage DNA (200 ng). Lane 2: cloned and amplified S1 segment of BTV-13. Lane 3: BTV-13 dsRNA (250 ng). Lane 4: cloned and amplified segment S1 from reovirus type 3. Lane 5: reovirus type 3 dsRNA (250 ng).

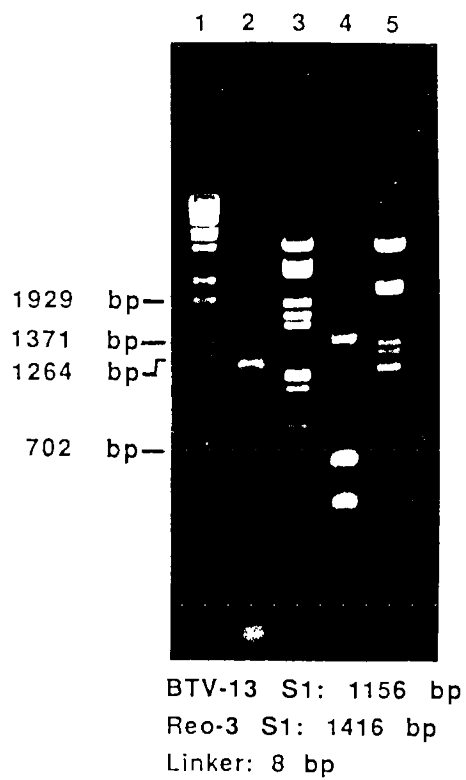
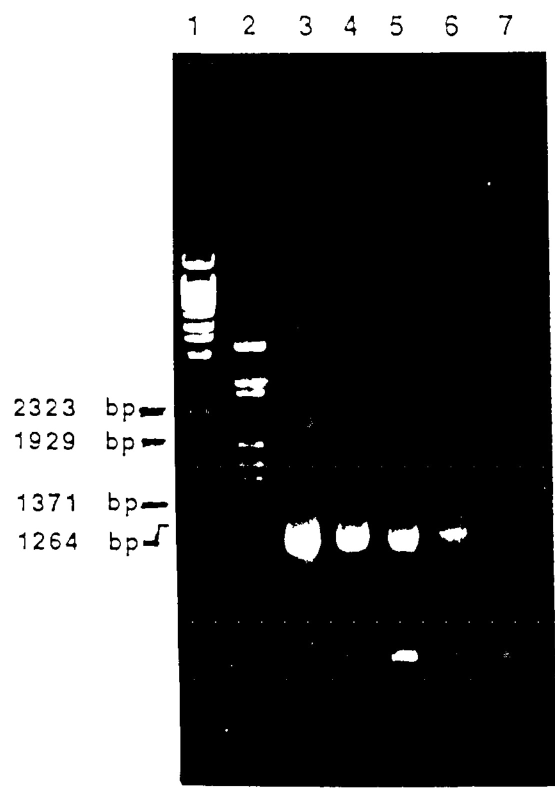

1 2 3 4 5
1929 bp
1371 bp
1264 bp
702 bp
BTV-13 S1: 1156 bp
Reo-3 S1: 1416 bp
Linker: 8 bp

Figure 3. Titration of template dsRNA used in ClampR reactions. Five-fold serial dilutions of genomic dsRNA were mixed with primers specific for the S1 segment of (A) BTV-13 or (B) reovirus type 3, denatured and reacted as described in (6) while using an annealing temperature of 35°C for each of the 40 amplification cycles. Aliquots of each reaction (20%) were analyzed by agarose gel electrophoresis. Lane 1: BstEII-digested lambda DNA (200 ng). Lane 2: BTV-13 dsRNA (250 ng). Starting dsRNA concentrations were: 300 ng (lane 3), 60 ng (lane 4), 12 ng (lane 5), 2.4 ng (lane 6), and 0.48 ng (lane 7).


A
1
2
3
4
5
6
7
2323 bp
1929 bp
1371 bp
1264 bp

B

Prior cloning experiments have demonstrated that the vast majority of cDNA synthesized from BTV dsRNA is less than full-length (7). If it is assumed that all 25 pg of starting RNA template was made into complete-copy DNA, at least a 50,000-fold amplification has occurred based on the yield in Fig. 3, lane 6. The actual value is much higher. Optimal yields of full-length DNA with minimal incomplete copy bands were recovered after 30 to 40 amplification cycles (Fig. 4).

The terminal sequences of specific BTV dsRNA segments are conserved among the serotypes, as is the case with most members of Reoviridae (8). Therefore, a pair of primers specific for the termini of each set of cognate dsRNA segments (e.g. the S1 segments of all five serotypes; 4) was used to clone and amplify that segment from total, genomic dsRNA of each of the five U.S. BTV serotypes. All 50 dsRNA segments could be readily cloned and amplified with representative results shown in Fig. 5. With all but the largest segments, which are between 3000 and 4000 base pairs, full-length DNAs are observed as single, discrete bands. The full-length cloning of these 50 dsRNA segments represents the conversion of approximately 100,000 bp of dsRNA into cDNA using less than 25 μg of starting dsRNA for all of the ClampR reactions.

To verify that clones made by the ClampR procedure resulted in full-length DNA, a clone of segment S1 from

Figure 4. Optimization of the number of ClampR amplification cycles. BTV-13 dsRNA, at a starting concentration of 300 ng (A) or 60 ng (B), was mixed with S1-specific primers, the S1 segment reverse transcribed, and then amplified for 20, 30, 40, 50, or 60 cycles. Aliquots (20%) of the reaction products were compared by agarose gel electrophoresis. The unmarked lane contains BTV-13 dsRNA (250 ng).

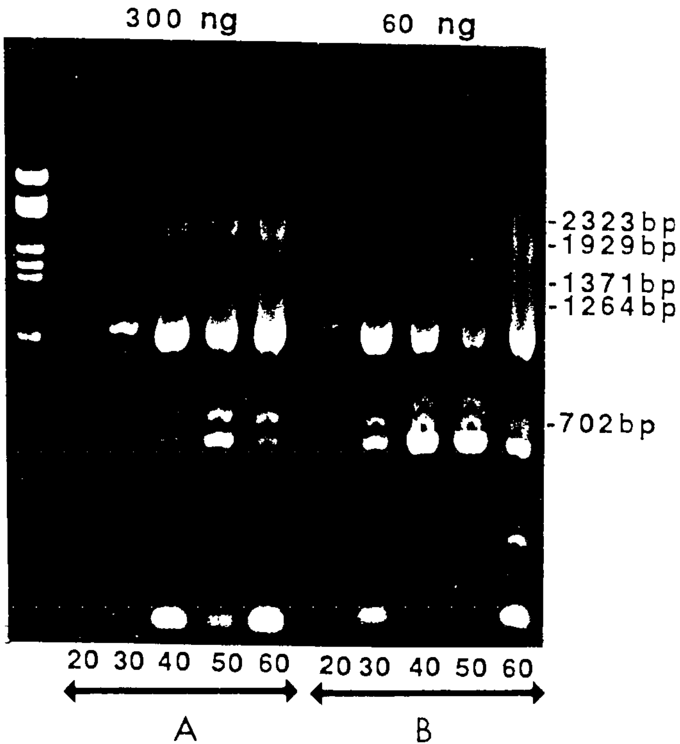
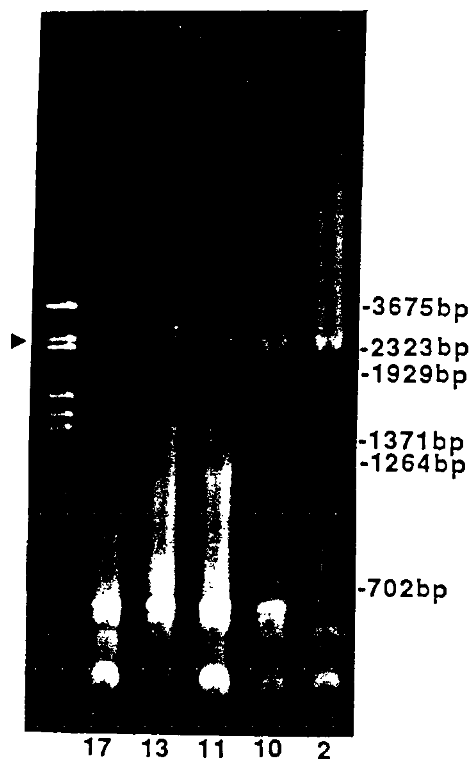

300 ng
60 ng
-2323bp
-1929bp
-1371bp
-1264bp
-702bp
20 30 40 50 60
20 30 40 50 60
A
B

Figure 5. Analysis of ClampR reaction products representing each size class (L, M, or S) of BTV genomic dsRNA. Primers specific for segment (A) L3, (B) M2, or (C) S3 were mixed with 300 ng of total genomic dsRNA from BTV-2, 10, 11, 13, or 17. cDNA was synthesized and amplified through 35 cycles. A 10% aliquot of each reaction product was analyzed by agarose gel electrophoresis.

A



B

C

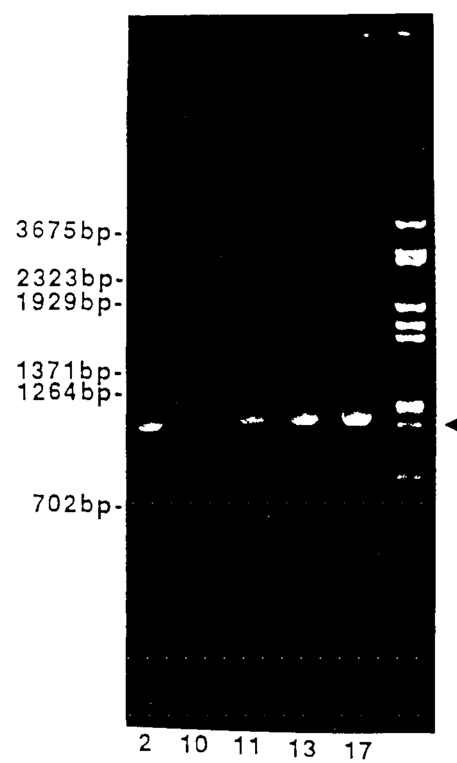


Figure 6. Nucleotide and deduced amino acid sequence of segment S1 of BTV-11 using ClampR-derived DNA. The initiation and termination codons of the long open reading frame are in bold print and an internal PstI site that produced 470 bp and 686 bp subfragments is underlined. Oligodeoxynucleotides specific for the termini of the S1 segment containing PstI sites at their 5' ends were used to prime cDNA synthesis and to amplify (40 cycles) any resulting full-length DNA copies of this segment starting from 300 ng of total genomic dsRNA. The resulting product was cut with PstI, which produced fragments of 470 bp and 686 bp. The fragments were separated by electrophoresis, ligated into pUC18, and inserted into E. coli (JM109) cells (6).

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GTTAAAAATCTGTAGAG ATG GAC ACT ATC GCT GCA AGA GCG CTC ACT GTG ATG CGA GCA     59
                   M   D   T   I   A   A   R   A   L   T   V   M   R   A

TGC GCT ACG CTT CAA GAA GCA AGA ATT GTG TTG GAA GCC AAT GTG ATG GAA ATT CTA GGG     119
 C   A   T   L   Q   E   A   R   I   V   L   L   A   N   V   M   E   I   L   G

ATA GCT ATC AAC AGG TAC AAT GGG CTC ACT CTA CGA GGA GTG ACG ATG CGC CCG ACC TCG     179
 I   A   I   N   R   Y   N   G   L   T   L   R   G   V   T   M   R   P   T   S

TTG GCA CAG AGA AAT GAG ATG TTT TTT ATG TGT TTG GAT ATG ATG CTG TCT GCT GCT GGG     239
 L   A   Q   R   N   E   M   F   F   M   C   L   D   M   M   L   S   A   A   G

ATA AAT GTT GGA CCG ATA TCA CCA GAC TAT ACT CAA CAT ATG GCT ACA ATT GGT GTG TTA     299
 I   N   D   G   P   I   S   P   D   Y   T   Q   H   M   A   T   I   G   V   L

GCG ACA CCG GAA ATA CCT TTT ACA ACG GAA GCG GCG AAT GAA ATA GCA CGG GTG ACT GGG     359
 A   T   P   E   I   P   F   T   T   E   A   A   N   E   I   A   R   V   T   G

GAG ACT TCG ACA TGG GGG CCA GCG CGT CAG CCT TAT GGT TTT TTC CTT GAA ACT GAA GAA     419
 E   T   S   T   W   G   P   A   R   Q   P   Y   G   F   F   L   E   T   E   L

ACC TTT CAA CCT GGG AGA TGG TTC ATG CGT GCC GCT CAA GCG GTA ACT GCA GTA GTG TGC     479
 T   F   Q   P   G   R   W   F   M   R   A   A   Q   A   V   T   A   V   V   C

GGT CCG GAT ATG ATC CAG GTG TCA CTA AAT GCT GGA GCG AGA GGG GAT GTG CAA CAA ATA     539
 G   P   D   M   I   Q   V   S   L   N   A   G   A   R   G   D   V   Q   Q   I

TTT CAG GGT CGT AAT GAT CCT ATG ATG ATA TAT TTA GTG TGG AGG AGA ATC GAA AAT TTC     599
 F   Q   G   R   N   D   P   M   M   I   Y   L   V   W   R   R   I   E   N   F

GCT ATG GCG CAA GGT AAT TCA CAG CAA ACT CAA GCG GGT GTG ACT GTT AGT GTT GGT GGG     659
 A   M   A   Q   G   N   S   Q   Q   T   Q   A   G   V   T   V   S   V   G   G

GTA GAC ATG AGG GCG GGA CGC ATT ATA GCG TGG GAT GGA CAG GCC GCG TTA CAT GTG CAT     719
 V   D   M   R   A   G   R   I   I   A   W   D   G   Q   A   A   L   H   V   H

AAC CCA ACA CAA CAG AAT GCG ATG GTG CAA ATA CAG GTT GTG TTC TAT ATA TCT ATG GAT     779
 N   P   T   Q   Q   N   A   M   V   Q   I   Q   V   V   F   Y   I   S   M   D

AAA ACT TTA AAT CAG TAC CCC GCT TTG ACT GCC GAG ATT TTT AAT GTT TAC AGC TTC AGG     839
 K   T   L   N   Q   Y   P   A   L   T   A   E   I   F   N   V   Y   S   F   R

GAT CAC ACA TGG CAT GGG CTA AGA ACG GCG ATA TTA AAC AGA ACC ACA CTG CCG AAC ATG     899
 D   H   T   W   H   G   L   R   T   A   I   L   N   R   T   T   L   P   N   M

CTG CCA CCA ATC TTC CCA CCA AAT GAT CGA GAT AGT ATC TTA ACT CTT CTA CTT TTA TCT     959
 L   P   P   I   F   P   P   N   D   R   D   S   I   L   T   L   L   L   L   S

ACA CTT GCT GAT GTT TAC ACT GTT TTG AGG CCG GAA TTT GCG ATT CAC GGC GTA AAT CCG     1019
 T   L   A   D   V   Y   T   V   L   R   P   E   F   A   I   H   G   V   N   P

ATG CCG GGG CCG CTC ACA CGT GCT ATT GCA CGC GCC GCC TAT GTG TAG TCCACTTTGCACGGG     1082
 M   P   G   P   L   T   R   A   I   A   R   A   A   Y   V

TGTGGGTTACATATGCGGTGTGTCGGTTGTGGGAAATATGTGACCCATTCAAACGTCTCTTAGATTAAACTTAC     1156
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BTV-11 was digested with PstI, ligated into a plasmid vector, and sequenced (6; Fig. 6). Sequence analyses demonstrated that the resulting clones contain the conserved S1 termini, are collectively of the expected length of 1156 bp, and contain a 349 codon open reading frame which encodes VP7, the major core protein of BTV (9).

The ClampR technique described here is a major advance in obtaining full-length DNA clones from dsRNA. The procedure is rapid, requiring only an overnight reaction, compared with the months to years previously required to clone and identify full-length cDNA from dsRNA. The general applicability of ClampR will facilitate the cloning of dsRNAs from other sources such as plant and fungal viruses and should be adaptable to cloning specific single-stranded RNAs, such as mRNA, as well. The simplicity and generality of the ClampR procedure make it amenable to automation for cloning of large numbers of dsRNAs, such as the 10 segments from the other 19 BTV serotypes found worldwide. Since the procedure is rapid (the reaction conditions can be further shortened with a concomitant decrease in DNA yield) and requires minimal template RNA, ClampR should also be useful in diagnosing RNA virus infections from sources such as tissue biopsies or circulatory fluids.

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(1989).

6. Methods: BTV and reovirus were cultured and their dsRNA purified as described elsewhere (J.K.-K. Li *et al.*, *Virology*, 105, 41 (1980); T.F. Kowalik and J.K.-K. Li, *ibid.*, 158, 276 (1987); *op. cit.*, 172, 189 (1989)). The concept of combining cloning and amplification steps in a single reaction vessel was based on C. Goblet *et al.*, *Nuc. Acids Res.* 17, 2144 (1989) and applied here with major modifications. Essentially, 60 to 500 ng of total genomic dsRNA was mixed with primers (500 ng each) of at least 13 bases which were complementary to each of the 3' ends of the target gene segment. The sample, in a final volume of 30 µl, was boiled for 4 minutes to denature the dsRNA and quenched on ice. The nucleic acids were then combined with a 20 µl reaction mixture containing 5 µl of 10X buffer (100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.1% (w/v) gelatin), 1 µl of stock nucleotides (10 mM each of dATP, dCTP, dGTP, and dTTP), 0.5 µl (15 U) of avian myeloblastosis virus reverse transcriptase (AMV-RT), 0.5 µl (2.5 U) of Taq polymerase and 13 µl of water. The resulting solution was overlayed with 50 µl of mineral oil, and the ClampR reaction carried out in a Programmable Cycle Reactor (Ericomp Corp).

The primed, denatured RNA was reverse transcribed in an initial 60 minute incubation at 42°C. AMV-RT was

used for this report, but mouse Moloney leukemia virus reverse transcriptase produced reduced but comparable results (data not shown). Full-length DNA clones were amplified by denaturing the double-stranded nucleic acid complexes at 95°C for 2 minutes, followed by a primer binding step at 35°C for 2 minutes and primer elongation at 72°C for 10 minutes. The amplification steps were usually repeated for 30 to 40 cycles with the last cycle changed to 95°C for 2 minutes, 35°C for 2 minutes, and 72°C for 20 minutes.

After completion of the cloning/amplification reactions, the sample was extracted once with chloroform, ethanol precipitated, and pelleted by centrifugation. The pellet was resuspended in 20 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) and an aliquot examined by electrophoresis through 1% (w/v) agarose gels.

Most of the primers used in the ClampR reaction had a restriction endonuclease site (PstI) incorporated into the 5' end of the primer for insertion of the cDNA into a plasmid vector. Bands representing full-length DNA copies were identified by ethidium bromide staining of agarose gels (GTG-grade SeaPlaque low melting agarose; FMC). The cDNA contained in the gel slices was digested "in gel" and the DNA repurified through a second gel. A gel slice containing the cDNA was

melted, mixed with a previously digested plasmid vector (pUC18), ligated, and transformed into competent Escherichia coli cells. These "in gel" procedures were based on instructions provided by the agarose manufacturer (FMC). Recombinants containing cDNA representing the S1 segment of BTV-11 were sequenced using Sequenase (US Biochemicals) and dideoxy chemistry (S. Tabor and C.C. Richardson, Proc. Natl. Acad. Sci. USA 84, 4767 (1987)).

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CHAPTER IV

THE COMPLETE NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE OF THE GENE ENCODING THE MAJOR INNER CAPSID PROTEIN, VP7, OF UNITED STATES PROTOTYPE BLUETONGUE VIRUS SEROTYPE 17

The major inner capsid protein, VP7, is encoded by segment S1 of bluetongue virus (BTV) and confers group specificity (1). The complete nucleotide sequence of the double-stranded RNA (dsRNA) S1 segment of United States prototype serotype 17 has been determined from a full-length cDNA. This clone was generated by modification of the polymerase chain reaction (ClampR reaction; 2) and inserted into pUC18. The nucleotide sequence of both strands was obtained using Sequenase (3) and the terminal sequence confirmed by direct dsRNA sequencing (Fig. 1; 4). The S1 segment was determined to be 1156 bp and full length (4, 5). The plus sense RNA has a 5' noncoding region of 17 bases and an 89 base, 3' noncoding region preceded by a UAA translation termination codon. Segment S1 of BTV-17 encodes a protein (VP7) of 349 amino acids (Fig. 1).

References

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GUUAAAAAUCUAUAGAG AUG GAC ACU AUC GCU GCA AGA GCA CUC ACU GUG AUG CGA GCA 59
M D T I A A R A L T V M R A

UGU GCU ACG CUU CAA GAG GCA AGA AUU GUG UUG GAA GCC AAU GUG AUG GAA AUU CUG GGG 119
C A T L Q E A R I V L E A N V M E I L G

AUU GCU AUC AAU AGG UAC AAU GGA CUU ACU CUA CGA GGA GUG ACG AUG CGC CCA ACC UCG 179
I A I N R Y N G L T L R G V T M R P T S

UUA GCA CAA AGA AAU GAG AUG UUU UUU AUG UGU UUG GAU AUG AUG CUG UCU GCU ACU GGG 239
L A Q R N E M F F M C L D M M L S A T G

AUA AAU GUC GGA CCG AUA UCG CCA GAC UAU ACU CAA CAU AUG GCU ACG AUU GGU GUG CUA 299
I N V G P I S P D Y T Q H M A T I G V L

GCA ACA CCG GAA AUA CCU UUU ACA ACG GAA GCG GCG AAU GAA AUA GCU CGA GUG ACU GGG 359
A T P E I P F T T E A A N E I A R V T G

GAG ACU UCG ACA UGG GGG CCA GCG CGU CAG CCU UAU GGU UUU UUC CUU GAA ACU GAG GAA 419
E T S T W G P A R Q P Y G F F L E T E E

ACC UUC CAA CCU GGG AGA UGG UUC AUG CGC GCC GCU CAA GCA GUA ACU GCA GUA GUG UGC 479
T F Q P G R W F M R A A Q A V T A V V C

GGU CCG GAU AUG AUU CAG GUG UCA GUC AAU GCU GGA GCG AGA GGG GAU GUA CAA CAG AUA 539
G P D M I Q V S L N A G A R G D V Q Q I

UUU CAG GGU CGU AAU GAU CCU AUG AUG AUA UAU UUA GUG UGG AGG AGA AUC GAA AAC UUU 599
F Q G R N D P M M I Y L V W R R I E N F

GCG AUG GCG CAA GGU AAU UCA CAA CAA ACU CAA GCG GGU GUG ACU GUC AGU GUU GGU GGA 659
A M A Q G N S Q Q T Q A G V T V S V G G

GUU GAC AUG AGG GCG GGA CGC AUU AUA GCG AGG GAU GGG CAG GCC GCG CUG CAC GUG CAU 719
V D M R A G R I I A W D G Q A A L H V H

AAC CCA ACA CAA CAG AAU GCG AUG GUG CAA AUA CAG CUU GUG UUC UAU AUA UCU AUG GAU 779
N P T Q Q N A M V Q I Q V V F Y I S M D

AAG ACU UUA AAC CAG UAC CCC GCA UUG ACU GCU GAG AUU UUU AAU GUU UAC AGC UUC AGG 839
K T L N Q Y P A L T A E I F N V Y S F R

GAU CAC ACA UGG CAU GGG CUA AGA ACG GCA AUA UUA AAC AGA ACC ACA CUG CCA AAC AUG 899
D H T W H G L R T A I L N R T T L P N M

CUG CCA CCA AUU UUC CCA CCA AAC GAU CGA GAU AGU AUC UUA ACU CUC CUA CCU UUA UCA 959
L P P I F P P N D R D S I L T L L P L S

ACA CUU GCU GAU GUU UAC ACU GUU UUA AGG CCG GAA UUU GCG AUU CA GGC GUA AAU CCG 1019
T L A D V Y T V L R P E F A I H G V N P

AUG CCA GGG CCG CUC ACA CGU GCU AUU GCA CGC GCC GCC UAU GUG UAA UCCACUUUGCACGGG 1079
M P G P L T R A I A R A A Y V

UGUGGGUUACAUAUGCGGUGUGUCGGUUGUGGGAAAUAUGUAACCCAUUUAAACGUCUCUUAGAUUACAGUUAC 1156

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CHAPTER V

BLUETONGUE VIRUS EVOLUTION: SEQUENCE ANALYSES OF GENOME SEGMENT S1 AND MAJOR CORE PROTEIN VP7

ABSTRACT

The S1 segments, encoding the group antigen, VP7, from the five United States prototype BTV serotypes were cloned in their entirety. The nucleotide and deduced amino acid sequence of segment S1 of BTV-2 was determined and compared with BTV-10, 11, 13, and 17. This completes the sequencing of this segment from the five United States BTV serotypes. Each segment is 1156 bp long and contains an open reading frame encoding the 349 amino acid VP7 protein. The vast majority (>94%) of the amino acids of VP7 among the serotypes are conserved, including the location (position 255) of a single lysine residue. Secondary structure analyses predict an eight-stranded ß-barrel between amino acid position 150 to 250 for each protein. This structure is similar to that observed in ssRNA viruses. The VP7 gene is flanked by conserved 5' and 3' noncoding regions. Stem-loop structures are predicted at the 3' end of each gene (nucleotide position 1058-1097). The S1 segments of BTV-2, 10, 11, and 17 have >93% of their nucleotides conserved, while <80% of their nucleotides are identical with BTV-13. Analyses of nucleotide mismatches in the third codon of the VP7 open reading frame, transition

frequencies, and evolutionary distances show that BTV-13 is the most distantly related serotype of the five and that BTV-10 and 17 are the closest related serotypes. Evolutionary distance calculations of segment L2 from BTV-10, 11, and 17 confirm the relatedness of BTV-10 and 17. Comparison with hybridization data on segment M3, which codes for VP5, suggested that BTV-17 evolved by a combination of genetic drift and genomic reassortment. The data also show that the five BTV serotypes found in the United States are derived from two distinct gene pools.

INTRODUCTION

Bluetongue virus (BTV) is the prototype *Orbivirus* in the Reoviridae family. As a model for the Reoviridae, studies of the relationships among BTV serotypes are necessary to better understand the genome organization, protein structure, genetic relatedness, and evolutionary relationships of members of this family. The United States has had limited exposure to influxes of BTV and accurate records have been kept of the isolation and characterization of different BTV isolates. This provides a unique opportunity to analyze samples of well characterized BTV isolates and, by deducing the phylogeny of these prototypes, determine the number of gene pools (sources) that have provided new serotypes in the United States. Consequently, measures to minimize the risk of new introductions may eventually be developed and tested.

Of the 24 BTV serotypes known, five (BTV-2, 10, 11, 13 and 17) are found in the United States. The relationships among these five serotypes have been investigated by genetic and serological means (Walton, 1980; Sugiyama et al., 1981; 1982; Rao et al., 1983; Squire et al., 1986; Huismans and Cloete, 1987; Kowalik and Li, 1987; 1989). To further develop our understanding of these relationships, sequence analyses of one of the genomic dsRNA segments was undertaken.

Segment S1, which encodes the group-specific antigen, VP7, and is the major core protein of BTV (Huismans and Erasmus, 1981), is the best representative of the 10 dsRNA segments comprising the BTV genome (Kowalik and Li, 1989). In addition to establishing a pattern of evolutionary divergence of BTV in the United States, analysis of the deduced amino acid sequence and structure/function predictions of VP7 will provide a better understanding of the relationships of BTV with other viruses.

We present the application of the ClampR protocol (Kowalik et al., 1990b) to clone the S1 segments of the five BTV serotypes found in the United States. The sequence of segment S1 from BTV-2 and the deduced amino acid sequence of VP7 are compared with their equivalents from BTV-10, 11, 13, and 17 (Yu et al., 1988; Kowalik and Li, 1989; Kowalik et al., 1990a; 1990b). This completes the sequencing of the S1 segments from the five United States BTV serotypes. Structural predictions of both RNA

and protein suggest strong conservation of stem-loops and ß-barrels, respectively. Analyses of evolutionary divergence show that four of the serotypes are very closely related even though two of them have been separated for at least 29 years. Temporal information was used to estimate a maximum evolutionary rate for segment S1. These data demonstrate that the five United States BTV serotypes are derived from two distinct gene pools.

MATERIALS AND METHODS

Virus culture and purification

United States prototype BTV-2 (Ona-B), 10, 11, 13, and 17 were obtained from the Arthopod-borne Animal Diseases Research Laboratory (Laramie, Wyoming) and plaque-purified three times in mouse L cells.

Virus growth in BHK cells and purification by sucrose gradient centrifugation have been described elsewhere (Kowalik and Li, 1987, 1989).

Cloning and sequencing of dsRNA segment S1

Total genomic dsRNA was released from BTV virions and purified as described (Kowalik and Li, 1989). The S1 segment was selectively cloned from genomic dsRNA of each BTV serotype using the ClampR procedure (Kowalik et al., 1990b). Briefly, BTV dsRNA (300 ng) was combined with 500 ng each of primers complementary with the conserved 3' ends of the S1 segments (Kiuchi et al., 1983). These primers

contained 7 bases of spacer sequence at the 5' end followed by a PstI site and 13 or 17 bases of BTV-specific sequence (AGTCGACCTGCAGGTAAGTGTAATCT and AGTCGACCTGCAGGTTAAAAATCTATAGAG, respectively). The same pair of primers were used to clone segment S1 from each BTV serotype. The volume of the genomic dsRNA and primers mixture was brought to 30 µl. The samples were boiled for 4 min and quenched on ice. A reaction mixture (20 µl) containing 5 µl of 10X buffer (100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.1% gelatin), 1 µl of the four dNTPs (10 mM each), 0.5 µl (15 units) of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), 0.5 µl (2.5 units) of Taq polymerase (Cetus), and 13 µl of water was then added to the cooled nucleic acid solution. This ClampR reaction mix was placed in a thermalcycler and incubated for 60 min at 42° to synthesize cDNA. Full-length DNA clones were then selectively amplified by PCR (polymerase chain reaction; Saiki et al., 1988) cycling (2 min at 95°, 2 min at 35°, and 10 min at 72°).

An aliquot (10%) of each ClampR reaction was examined by agarose gel electrophoresis to confirm the production of full-length cDNA. The remainder of the samples were extracted with phenol/chloroform (1:1), ethanol precipitated and pelleted by centrifugation. The pellets were resuspended in 20 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA) and the full-length cDNA isolated by electrophoresis through a 1% (w/v) agarose (SeaKem GTG-grade agarose, FMC)

minigel. Bands representing the cDNA were excised from the gel, the cDNA eluted and concentrated by ethanol precipitation. The cDNA samples were digested with PstI and purified through low-gelling agarose (SeaPlaque GTG-grade agarose, FMC). The DNAs were then excised from the gel and ligated into pUC18 which had been digested with PstI and treated with calf-intestinal phosphatase (Boehringer Mannheim). The ligation products were transformed into competent Escherichia coli JM109 cells and screened for appropriate selection criteria. The ligation and transformation steps were done "in gel" according to the methods provided by the agarose manufacturer.

Plasmid DNA was isolated from recombinant bacteria containing BTV S1 sequences and the nucleotide sequence determined (Kraft et al., 1988). Both strands of the BTV clones were sequenced in their entirety. To confirm that the ends of the cDNA were accurate, the termini of dsRNA segment S1 from each BTV serotype was also sequenced using reverse transcriptase and terminal deoxynucleotide transferase (Kowalik and Li, 1989).

Phylogenetic analysis

Evolutionary distances (K) were calculated by comparisons of transitions and transversions among the BTV serotypes (Kimura, 1980). The values were determined using the equation

$$K = -1/2 \ln ((1-2P-Q) \times (1-2Q)^{1/2}),$$

where P is the fraction of nucleotide sites in transitions and Q is the fraction of nucleotide sites in transversions.

A phylogenetic tree was constructed using the distance matrix method of Fitch and Margoliash (1967) as contained in the Phylip package of Joseph Felsenstein (University of Washington, Seattle).

RESULTS

Cloning of BTV genome segment S1

First strand DNA synthesis of segment S1 took place during the initial 60 min incubation at 42° as reported for reovirus (Cashdollar et al., 1982). When the thermalcycler switched into the PCR cycling mode, the reverse transcriptase and template RNA degraded while the ssDNA produced in the cloning reaction was made double-stranded and full-length cDNA amplified. All five S1 segments have been cloned with similar efficiencies by using the same pair of primers derived from the terminal sequence of BTV-11 (Kiuchi et al., 1983). Initial attempts to blunt-end ligate ClampR products directly into cloning vectors were unsuccessful, presumably due to modification of the 5' ends of the primers or synthesized DNA during amplification (data not shown). It was therefore necessary to incorporate a restriction endonuclease site upstream of the complimentary BTV primer sequences. The placement of a restriction site several bases internal to this 5' modification was also required for efficient cutting by the

enzyme (data not shown).

The five United States BTV serotypes were cloned into full-length cDNA using the ClampR procedure (Kowalik et al., 1990b). Fig. 1 shows aliquots of clones produced by the ClampR reaction of segment S1 from each BTV serotype. The cDNAs tend to migrate slower than their dsRNA counterpart. This phenomenon becomes more pronounced as the length of the starting dsRNA segment is reduced (Kowalik et al., 1990b; data not shown). Upon digestion of the cDNA with PstI, fragments of 470 bp and 686 bp were observed for BTV-2, 10, 11, and 17, whereas BTV-13 cDNA remained intact (data not shown). Each of the DNA fragments were cloned into the PstI site of pUC18 and their nucleotide sequence determined. Sequencing of ClampR products derived from segment S1 of BTV-11 show that these two PstI-produced cDNAs fragments together represent a full-length S1 clone (Kowalik et al., 1990b).

Nucleotide and deduced amino acid sequencesof BTV segment S1 and VP7 from the five BTV serotypes

The nucleotide sequence of United States prototype BTV-2 segment S1 and alignment with the other four United States prototype BTV serotypes is shown in Fig. 2. Each S1 segment is 1156 bp long and contains an open reading frame (ORF) capable of encoding a protein of 349 amino acids. This is followed by a single termination codon. This ORF begins at nucleotide position 18 and ends at position 1064.

Figure 1. Cloning and amplification of dsRNA (ClampR) segment S1 from the five United States BTV serotypes. Total genomic BTV dsRNA was mixed with primers complimentary to the 3' end of the S1 segment, converted into cDNA and amplified by PCR. One-tenth of each reaction was analyzed by agarose gel electrophoresis. The numbers beneath each lane denote the BTV serotype of the starting dsRNA. The unmarked lane is BTV genomic dsRNA (250 ng).

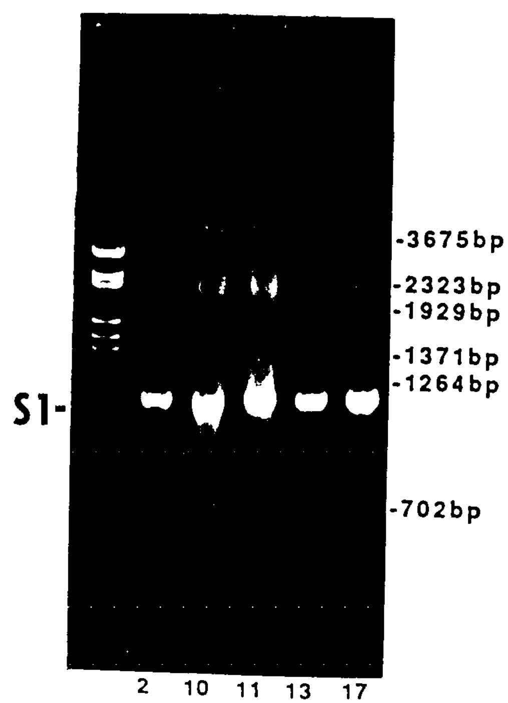
-3675bp
-2323bp
-1929bp
-1371bp
-1264bp
S1-
-702bp
2
10
11
13
17

Figure 2. Nucleotide sequence of the plus strand of segment S1 from the five United States BTV serotypes. The complete sequence of BTV-2 and the mismatches of BTV-10, 11, 13, and 17 are shown. The start and stop codons are underlined. Asterisks denote bases identical with BTV-2.

```
BTV- 2      GTTAAAAATCTATAGAG ATG GAC ACT ATC GCT GCA AGA GCA CTC ACT GTG ATG CGA GCA     59
     10     ................. ... ... ... ... ..C ... ... ... ... ... ... ... ... ...
     11     ................. ... ... ... ... ... ... ... ..G ... ... ... ... ... ...
     13     ............C.... ... ... ... ... ..A ... ... ..T ... ... ... ... ..G .T.
     17     ................. ... ... ... ... ... ... ... ... ... ... ... ... ... ...

  2 TGT GCT ACG CTT CAA GAA GCA AGA ATT GTG TTG GAA GCT AAT GTG ATG GAA ATA CTG GGG   119
 10 ... ... ... ... ... ..G ... ... ... ... ... ... ..C ... ... ... ... ..T T.. ...
 11 ..C ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ..T ..A ...
 13 ... ... ..C T.A ... ..G ..C C.. ... ..C C.A ... C.C ... ..A ... ..G ... ... ..A
 17 ... ... ... ... ... ..G ... ... ... ... ... ... ..C ... ... ... ... ..T ... ...

  2 ATA GCT ATC AAC AGG TAC AAT GGA CTA ACT CTA CGT GGG GTA ACG ATG CGC CCG ACC TCG   179
 10 ... ... ... ..T ... ... ... ... ..C ... T.. ..A ..A ..G ... ... ... ... ... ...
 11 ... ... ... ... ... ... ... ..G ..C ... ... ..A ..A ..G ... ... ... ... ... ...
 13 ... ..A ... ..T ..A ..T ... ... T.. ... ... ... ..A ..T ... ... A.A ..C ..T ..A
 17 ..T ... ... ..T ... ... ... ... ..T ... ... ..A ..A ..G ... ... ... ..A ... ...

  2 TTA GCG CAA AGA AAT GAG ATG TTT TTT ATG TGT CTT GAT ATG ATG TTG TCT GCT GCG GGA   239
 10 ... ..A ... ... ... ... ... ... ... ... ... T.G ... ... ... C.. ... ... ..T ..T
 11 ..G ..A ..G ... ... ... ... ... ... ... ... T.G ... ... ... C.. ... ... ..T ..G
 13 ... ..T ... ... ... ..A ... ... ..C ... ... T.A ... ... ... G.. ..A ..A ... ...
 17 ... ..A ... ... ... ... ... ... ... ... ... T.G ... ... ... C.. ... ... A.T ..G

  2 ATA AAT GTT GGA CCG ATA TCG CCA GAT TAT ACT CAA CAT ATG GCT ACA ATT GGT GTA CTA   299
 10 ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ..G ... ... ... ...
 11 ... ... ... ... ... ... ..A ... ..C ... ... ... ... ... ... ... ... ... ..G T..
 13 ... ... ..C ... ..A ..C ..A ..C ..T ... ..G ... ... ... ... ..T ..A ... ..G ..G
 17 ... ... ..C ... ... ... ... ... ..C ... ... ... ... ... ... ..G ... ... ..G ...

  2 GCA ACG CCG GAA ATC CCT TTT ACA ACA GAA GCG GCG AAT GAA ATA GCA CGC GTG ACT GGG   359
 10 ... ..A ... ... ..A ... ... ... ..G ... ... ... ... ... ... ..T ..A ... ... ...
 11 ..G ..A ... ... ..A ... ... ... ..G ... ... ... ... ... ... ... ..G ... ... ...
 13 ... ... ..T ..G ..A ..G ..C ... ..T ..G ..A ... ... ..G ..T ..T ..T ..T ..A ..A
 17 ... ..A ... ... ..A ... ... ... ..G ... ... ... ... ... ... ..T ..A ... ... ...

  2 GAG ACT TCG ACA TGG GGG CCG GCG CGT CAG CCT TAT GGT TTT TTC CTT GAA ACT GAG GAG   419
 10 ... ... ... ... ... ... ..A ... ... ... ... ... ... ..C ... ... ... ... ... ..A
 11 ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ..A ..A
 13 ... ... ..A ... ... ..A ... ... ... ... ... ..C ..A ... ... ... ... ... ..A ...
 17 ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ..A

  2 ACT TTC CAA CCT GGA AGA TGG TTC ATG CGC GCC GCT CAA GCG GTA GCT GCA GTG GTG TGC   479
 10 ..C ... ... ..A ..G ..G ... ... ... ... ... ... ... ..A ... A.. ... ..A ... ...
 11 ..C ..T ... ... ..G ... ... ... ... ..T ... ... ... ... ... A.. ... ..A ... ...
 13 GT. .AT ... ... ... ... ... ... ..A ..T ... ... ... .TT ..T A.A C.. ... ..T ..T
 17 ..C ... ... ... ..G ... ... ... ... ... ... ... ... ..A ... A.. ... ..A ... ...

  2 GGT CCG GAT ATG ATT CAG GTG TCA CTA AAT GCT GGA GCG AGA GGG GAT GTA CAA CAG ATA   539
 10 ... ... ... ... ... ..A ... ... ..T ... ... ... ... ... ..A ... ... ... ... ...
 11 ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ..G ... ..A ...
 13 ..A ..A A.. ... G.. ... ..T ... ..T ... ... ... ... .T. ..T ... ..G ..G ... ..C
 17 ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ...

  2 TTT CAG GGT CGT AAT GAT CCC ATG ATG ATA TAT TTG GTA TGG AGA AGA ATC GAA AAC TTT   599
 10 ... ... ... ... ... ... ... ... ... ... ... ..A ..G ... ..G ... ... ... ... ...
 11 ... ... ... ... ... ... ..T ... ... ... ... ..A ..G ... ..G ... ... ... ..T ..C
 13 ... ..A ... ... ... ... ... ... ... ..T ... ..A ..T ... ... C.G ... ..G ... ..C
 17 ... ... ... ... ... ... ..T ... ... ... ... ..A ..G ... ..G ... ... ... ... ...

  2 GCG ATG GCG CAA GGT AAT TCA CAG CAA ACT CAA GCA GGT GTG ACC GTT AGC GTT GGT GGA   659
 10 ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ..T ..C ..T ... ... ...
 11 ..T ... ... ... ... ... ... ... ... ... ... ..G ... ... ..T ... ..T ... ... ..G
 13 T.C ... C.. ..G ... ... ... ... .GC ..G TT. ..T ..C ..C ..T ..G ..T ..G ... ..T
 17 ... ... ... ... ... ... ... ..A ... ... ... ..G ... ... ..T ..C ..T ... ... ...

  2 GTT GAC ATG AGA GCG GGA CGC ATT ATA GCG TGG GAT GGA CAG GCC GCA TTG CAT GTG CAT   719
 10 ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ..G C.. ... ... ...
 11 ..A ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ..G ..A ... ... ...
 13 ..G ..T ... ... ... ..G ... ..A ..C ..A ... ... ..G ... ..G .TG C.A ..A A.T ..C
 17 ... ... ... ..G ... ... ... ... ... ... ... ... ..G ... ... ..G C.. ..C ... ...

  2 AAC CCT ACA CAA CAG AAT GCG ATG GTG CAG ATA CAG GTT GTG TTC TAT ATA TCT ATG GAT   779
 10 ..T ..G ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ...
 11 ... ..A ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ...
 13 ..T ..G ..T ... ..A ... ..T ... ..A ..A ..T ... ..G ... ..T ..C G.T ..C ... ...
 17 ... ..A ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ...

  2 AAA ACT TTA AAC CAG TAC CCA GCT TTG ACT GCC GAA ATT TTT AAT GTT TAC AGC TTC AGG   839
 10 ... ... ... ... ... ... ..C ... ... ... ..T ..G ... ..C ... ... ... ... ... ...
 11 ... ... ... ..T ... ... ..C ... ... ... ... ..G ... ... ... ... ... ... ... ...
 13 ..G ..C C.C ... ... ... ..C ..A C.A ..G ..A ... ..A ... ... ..G ... ... ..T ..A
 17 ..G ... ... ... ... ... ..C ... ... ... ..T ..G ... ... ... ... ... ... ... ...

  2 GAT CAT ACA TGG CAT GGA TTA AGA ACG GCA ATA TTA AAC AGA ACC ACA CTG CCT AAC ATG   899
 10 ..C ..C ... ... ... ..G C.. ... ... ..G ... ... ... ... ... ... ... ..A ... ...
 11 ... ..C ... ... ... ..G C.. ... ... ..G ... ... ... ... ... ... ... ..G ... ...
 13 ..C ..C ..T ... ... ... C.G ..G ..C ..T ... C.. ... ... ..A ..G ..T ..A ..T ...
 17 ... ..C ... ... ... ..G C.. ... ... ... ... ... ... ... ... ... ... ..A ... ...

  2 CTG CCA CCA ATC TTC CCA CCA AAT GAT CGT GAT AGT ATC TTA ACC CTT CTA CTT TTA TCT   959
 10 ... ... ... ... ... ... ... ... ... ..A ... ..C ... ... ..T ... ... ... ... ...
 11 ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ..T ... ... ... ... ...
 13 T.A ..T ... ... ..T ..G ..G ... ..C ..G ..C ... G.A C.C ..G A.. ..G ..A C.G ..G
 17 ... ... ... ..T ... ... ... ..C ... ..A ... ... ... ... ..T ..C ... .C. ... ...

  2 ACA CTC GCT GAT GTT TAC ACT GTG TTA AGA CCA GAG TTT GCG ATT CAC GGC GTA AAT CCG  1019
 10 ... ..T ... ... ... ... ... ..T ... ..G ... ... ... ... ... ... ... ... ... ...
 11 ... ..T ... ... ... ... ... ..T ..G ..G ..G ..A ... ... ... ... ... ... ... ...
 13 ..G ... ..A ... ... ..T T.A ..T ..G ... ..T ... ... ..A ..C ... ... ... ... ..A
 17 ... ..T ... ... ... ... ... ..T ... ..G ..G ..A ... ... ... ... ... ... ... ...

  2 ATG CCA GGG CCG CTC ACA CGT GCT ATT GCA CGC GCC GCC TAT GTG TAGTCCACTTTGCACGGGT  1082
 10 ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ...................
 11 ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ...................
 13 ... ..T ..C ... ..T ... ... ... ..G ... ..T ... ..T ..C .CA ...............T...
 17 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A.................

  2 GTGGGTTACATATGCGGTGTGTCGGTTGTGGGAAATATGTGACCCATTTAAACGTCTCTTAGATTACACTTAC  1156
 10 ........................................A................................
 11 ................................................C........................
 13 ........TGCGG.T.............CAA.........AT.TTG.............GG............
 17 ........................................A................................
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There is an 18 base 5' and an 89 base 3' noncoding region in each segment. There are no deletions or insertions in any of the S1 sequences.

The deduced amino acid sequence of VP7 from BTV-2 and comparisons with VP7 from BTV-10, 11, 13, and 17 are shown in Fig. 3 and the amino acid composition of each is shown in Table 1. The proteins each contain 349 amino acids. The average MW of VP7 is approximately 38,550, with VP7 from BTV-2 and 13 having the lowest and highest MW, respectively. There are 25 acidic, 27 basic, and 6 histidine residues in VP7 from BTV-2, 10, 11, and 17, while BTV-13 has 25 acidic, 26 basic, and 5 histidine residues. Thus VP7 has a slight positive charge (+1.0 to +1.5) at pH 7.0. VP7 contains only one lysine residue which is conserved at position 255 in all five serotypes. The biological significance of this observation is not known, but the existence and specific location of this residue has been experimentally confirmed (Yang et al., 1990). The proteins are very well conserved with 0 (BTV-10:11) to 22 (BTV-10:13 and BTV-11:13) amino acid differences between the serotypes (Table 3). BTV-2, 10, 11, and 17 have greater than 99% of their VP7 amino acids in identical matches, while BTV-13 has consistently less than 94% identity.

DISCUSSION

Segment S1 of the five United States prototype BTV

Figure 3. Deduced amino acid sequence of VP7 from BTV-2 with differences among BTV-10, 11, 13, and 17 shown for comparison. Asterisks denote amino acids that are identical with VP7 from BTV-2. Conservative substitutions are identified by plain text and differences are in bold print. The single conserved lysine residue at position 255 is boxed.

```
BTV- 2  MDTIAARALT VMRACATLQE ARIVLEANVM EILGIAINRY   40
    10  ********** ********** ********** **********
    11  ********** ********** ********** **********
    13  ********** ***V****** ******P*** **********
    17  ********** ********** ********** **********

     2  NGLTLRGVTM RPTSLAQRNE MFFMCLDMML SAAGINVGPI   80
    10  ********** ********** ********** **********
    11  ********** ********** ********** **********
    13  ********** ********** *********V **********
    17  ********** ********** ********** **T*******

     2  SPDYTQHMAT IGVLATPEIP FTTEAANEIA RVTGETSTWG  120
    10  ********** ********** ********** **********
    11  ********** ********** ********** **********
    13  ********** ********** ********** **********
    17  ********** ********** ********** **********

     2  PARQPYGFFL ETEETFQPGR WFMRAAQAVA AVVCGPDMIQ  160
    10  ********** ********** *********T **********
    11  ********** ********** *********T **********
    13  ********** ****VY**** *******V*T P*****N*V*
    17  ********** ********** *********T **********

     2  VSLNAGARGD VQQIFQGRND PMMIYLVWRR IENFAMAQGN  200
    10  ********** ********** ********** **********
    11  ********** ********** ********** **********
    13  *******I** ********** ********** ****S*P***
    17  ********** ********** ********** **********

     2  SQQTQAGVTV SVGGVDMRAG RIIAWDGQAA LHVHNPTQQN  240
    10  ********** ********** ********** **********
    11  ********** ********** ********** **********
    13  **R*L***** ********** *********V *QI*******
    17  ********** ********** ********** **********

     2  AMVQIQVVFY ISMDKTLNQY PALTAEIFNV YSFRDHTWHG  280
    10  ********** ********** ********** **********
    11  ********** ********** ********** **********
    13  ********** V********* ********** **********
    17  ********** ********** ********** **********

     2  LRTAILNRTT LPNMLPPIFP PNDRDSILTL LLLSTLADVY  320
    10  ********** ********** ********** **********
    11  ********** ********** ********** **********
    13  ********** ********** ******V**I **********
    17  ********** ********** ********** *P********

     2  TVLRPEFAIH GVNPMPGPLT RAIARAAYV             349
    10  ********** ********** *********
    11  ********** ********** *********
    13  S********* ********** ********A
    17  ********** ********** *********
```

TABLE 1

Deduced Amino Acid Composition of VP7

Amino acid	BTV-2 VP7 residue #	BTV-2 VP7 residue %	BTV-10 VP7 residue #	BTV-10 VP7 residue %	BTV-11 VP7 residue #	BTV-11 VP7 residue %	BTV-13 VP7 residue #	BTV-13 VP7 residue %	BTV-17 VP7 residue #	BTV-17 VP7 residue %
A	41	11.7	40	11.5	40	11.5	34	9.7	39	11.2
R	24	6.9	24	6.9	24	6.9	24	6.9	24	6.9
D	13	5.2	13	5.2	13	5.2	12	5.4	13	5.2
N	18	3.7	18	3.7	18	3.7	19	3.4	18	3.7
C	3	0.9	3	0.9	3	0.9	3	0.9	3	0.9
E	14	5.7	14	5.7	14	5.7	14	5.4	14	5.7
Q	20	4.0	20	4.0	20	4.0	19	4.0	20	4.0
G	23	6.6	23	6.6	23	6.6	23	6.6	23	6.6
H	6	1.7	6	1.7	6	1.7	5	1.4	6	1.7
I	24	6.9	24	6.9	24	6.9	24	6.9	24	6.9
L	28	8.0	28	8.0	28	8.0	27	7.7	27	7.7
K	1	0.3	1	0.3	1	0.3	1	0.3	1	0.3
M	19	5.4	19	5.4	19	5.4	19	5.4	19	5.4
F	14	4.0	14	4.0	14	4.0	13	3.7	14	4.0
P	21	6.0	21	6.0	21	6.0	24	6.9	22	6.3
S	11	3.2	11	3.2	11	3.2	13	3.7	11	3.2
T	29	8.3	30	8.6	30	8.6	28	8.0	31	8.9
W	5	1.4	5	1.4	5	1.4	5	1.4	5	1.4
Y	9	2.6	9	2.6	9	2.6	10	2.9	9	2.6
V	26	7.4	26	7.4	26	7.4	32	9.2	26	7.4
Total	349	100	349	100	349	100	349	100	349	100
Charge		+1		+1		+1		+1.5		+1
Size	38,521		38,551		38,551		38,619		38,565	

serotypes has been successfully cloned from total genomic dsRNA using the ClampR protocol (Kowalik et al., 1990b). The entire process from genomic dsRNA isolation through transformation requires only 3 days to complete. DNA sequencing of resulting clones and direct dsRNA sequencing permitted alignment of the S1 sequences of the five serotypes (Fig. 2). Sequence analyses show there is an ORF (nucleotide position 18-1064) capable of coding for a protein of 349 amino acids (Table 2). There is another ORF which can encode a protein of 31 amino acids (position 694-786) in BTV-2, 10, 11, and 17 and extended by 6 codons (position 694-804) in BTV-13 (Table 2). Each of these ORFs retains the consensus sequence for eukaryotic initiation of protein synthesis (Kozak, 1986). There are no additional ORFs of greater than 20 codons conserved in all five serotypes. Although viral expression of the 349 codon ORF has been demonstrated in proteolytic studies of VP7 (Li et al., 1989; Yang et al., 1990), none of the other potential proteins encoded by segment S1 have been identified in BTV infections.

The hydropathy (Kyte and Doolittle, 1982) of VP7 from each serotype is well conserved, exhibiting a regular pattern of hydrophobic and hydrophilic stretches throughout much of the protein, suggesting a specific structure for VP7 (data not shown). VP7 from BTV-2, 11, and 17 maintains the eight-stranded ß-barrel predicted for VP7 of BTV-10 and 13 (Kowalik and Li, 1989). The ß-barrel is predicted to

TABLE 2

Open Reading Frames Summary of the S1 Segments of Five BTV Serotypes

Reading Frame	Nucleotides	Coding Capacity[a]	Serotype 2	10	11	13	17	Nucleotides	Coding Capacity	Serotype 2	10	11	13	17
A. Plus Strand								B. Minus Strand						
1	337-519	61				X		937-830	36		X			
	394-462	23	X	X	X		X	682-572	37					X
	694-786	31	X	X	X		X							
	694-804	37				X								
	922-984	21				X								
	1060-1122	21	X	X	X		X							
2	59-130	24		X		X	X							
	59-193	45			X									
	437-505	22				X								
	437-550	38	X		X		X							
	689-778	30				X								
	848-922	25		X	X									
	848-931	28					X							
3	18-1064	349	X	X	X	X	X	854-789	22	X				X
								856-789	23		X	X		
								719-621	33	X	X			X
								278-183	32	X	X			X

[a] Number of codons which encode an amino acid.

cover the region from approximately position 150 to 250 and contains the loop region observed in picornaviruses (Rossmann et al., 1985; Rossmann, 1988). This structure is significant since it is the main structural motif of all ssRNA viruses whose three dimensional structure is known. Its presence suggests a common origin of ssRNA viruses from plants and animals (Rossmann, 1988). The predicted conservation of a similar structure of the major core protein of BTV supports the contention that dsRNA viruses may share a common precursor with ssRNA viruses (Kowalik and Li, 1989). The presence of the putative eight-stranded ß-barrel also suggests development of pharmacological agents analogous to the WIN series used against picornaviruses as therapeutic agents for use against BTV infections (see Rossmann, 1988; Argos, 1989). The nucleotide makeup of each serotype is very similar with G and C residues representing 46.3% (BTV-2) to 47.0% (BTV-11) of the nucleotide residues. The S1 nucleotide sequence among the serotypes is generally well conserved with BTV-2, 10, 11, and 17 maintaining 93.6% (BTV-2:11) to 97% (BTV-10:17) identity (Table 3). Segment S1 of BTV-13 is more distantly related to this group with only 79.0% (BTV-11:13) to 79.8% (BTV-2:13) of the nucleotide sequence conserved. There is a difference of 208 matches between the most (BTV-10:17) and least (BTV-11:13) conserved sequence pairing. Most of the these nucleotide changes occur within the ORF where 35% of the codons have at least

TABLE 3

Relatedness Comparisons of S1 and VP7 of BTV Serotypes

	Serotype Pairing									
	2:10	2:11	2:13	2:17	10:11	10:13	10:17	11:13	11:17	13:17
A. Nucleotide Sequence										
Total Matches	1087/1156 (94.0%)	1082/1156 (93.6%)	923/1156 (79.8%)	1085/1156 (93.9%)	1100/1156 (95.2%)	920/1156 (79.6%)	1121/1156 (97.0%)	913/1156 (79.0%)	1104/1156 (95.5%)	919/1156 (79.5%)
Total Mismatches	69/1156 (6.0)%	74/1156 (6.4%)	233/1156 (20.2%)	71/1156 (6.1%)	56/1156 (4.8%)	236/1156 (20.6)%	35/1156 (3.0%)	243/1156 (21.0%)	52/1156 (4.5%)	237/1156 (20.5%)
Open Reading Frame (ORF) Matches	977/1046 (93.4%)	972/1046 (92.9%)	830/1046 (79.3%)	976/1046 (93.3%)	992/1046 (94.8%)	825/1046 (78.9%)	1012/1046 (96.7%)	821/1046 (78.5%)	995/1046 (95.1%)	827/1046 (79.1%)
ORF Mismatches	69/1046 (6.6%)	74/1046 (7.1%)	216/1046 (20.7%)	70/1046 (6.7%)	54/1046 (5.2%)	221/1046 (21.3%)	34/1046 (3.3%)	225/1046 (21.5%)	51/1046 (4.9%)	219/1046 (20.9%)
B. Amino Acid Sequence										
Matches	348/349 (99.7%)	348/349 (99.7%)	326/349 (93.4%)	346/349 (99.1%)	349/349 (100%)	327/349 (93.7%)	347/349 (99.4%)	327/349 (93.7%)	347/349 (99.4%)	325/349 (93.1%)
Mismatches	1/349 (0.3%)	1/349 (0.3%)	23/349 (6.6%)	3/349 (0.9%)	0/349 (0.0%)	22/349 (6.3%)	2/349 (0.6%)	22/349 (6.3%)	2/349 (0.6%)	24/349 (6.9%)

one base substitution. The 5' and 3' termini of the coding strand are more conserved with just a single nucleotide difference (BTV-13) in the 5' noncoding region (Fig. 2). Only BTV-13 and 17 have differences in the 3' noncoding region.

The sequences of the segment S1 plus strand near the translational termination codon have the potential to form stable stem-loop structures. This includes the last few codons and part of the 3' noncoding region (nucleotide position 1058 to 1097; Kowalik and Li, 1989). BTV-2 and 11 have predicted stem-loops identical to BTV-10. The stems are 14 bp long with an 11 base loop (ΔG = -14.6 kcal/mol; data not shown). The stem-loop structure of BTV-17 is similar to BTV-2, 10, and 11 except for a G>A transition within the stop codon of BTV-17 slightly reducing the free energy of stabilization (ΔG = -14.3 kcal/mol). The stem of this predicted structure in BTV-13 is 2 bp shorter than the others but is more stable (ΔG = -18.9 kcal/mol).

Even though the significance of the stem-loop structures is not known, their presence in the 3' ends of the plus strand of dsRNA viruses and ssRNA viruses indicate that they may be a ubiquitous feature of RNA viruses. The presence of stem-loop structures at the 3' end of viral RNAs have been predicted or experimentally demonstrated in diverse groups such as BTV (Kowalik and Li, 1989), reovirus (Li _et al._, 1980), several tymoviruses (Reitveld _et al._, 1982; van Belkum _et al._, 1987; 1988; Ding _et al._, 1989;

Osorio-Keese et al., 1989), cucumber mosaic virus (Rizzo and Palukaitis, 1989), Punta Toro virus (Emery and Bishop, 1987), and L-A dsRNA virus of yeast (Esteban et al., 1989). While the function(s) of these structures is unknown in most cases, Esteban et al., (1989) demonstrated that a 3' stem-loop is a cis-acting element required in the replication of plus strand ssRNA into dsRNA for L-A dsRNA virus of yeast. A cis-acting region in the 3' untranslated region of an avian sarcoma virus required for viral RNA replication has also recently been identified (Hirano and Wong, 1989). In cases where viral RNA genomes are segmented, such as BTV, it has been suggested that these structures might serve as recognition signals and nucleation sites for the sorting of each unique genomic segment into a maturing viral particle (Kowalik and Li, 1989).

The difference between conservation of sequence at the nucleotide and amino acid level can be explained by analyzing the location of nucleotide substitutions within the codons of the ORF (Table 4). A base in the first or second codon position has a 0.2% to 2.7% and a 0.0% to 0.9% frequency of mismatch, respectively, whereas the third codon position has a mismatch frequency of 3.0% to 18.4%. We have observed that 83.3% (BTV-10:13) to 96.3% (BTV-10:11) of nucleotide changes occur in the third codon position of the ORF. Most of these differences do not result in amino acid substitutions. Comparisons of BTV-13

TABLE 4

Comparison of Nucleotide Mismatches by Codon Position of Segment S1 Among the Five U.S. BTV Serotypes

	Serotype Pairing									
	2:10	2:11	2:13	2:17	10:11	10:13	10:17	11:13	11:17	13:17
A. Number and percentage of mismatches in each codon position[a]										
All codon positions	69	74	216	70	54	221	34	225	51	219
First base codon position	6 (0.6%)	4 (0.4%)	27 (2.6%)	6 (0.6%)	2 (0.2%)	29 (2.7%)	2 (0.25%)	25 (2.4%)	2 (0.2%)	23 (2.2%)
Second base codon position	0 (0.0%)	0 (0.0%)	8 (0.8%)	1 (0.1%)	0 (0.0%)	8 (0.8%)	1 (0.1%)	8 (0.8%)	1 (0.1%)	9 (0.9%)
Third codon position	63 (6.0%)	70 (6.7%)	181 (17.3%)	63 (6.0%)	52 (5.0%)	184 (17.6%)	31 (3.0%)	192 (18.4%)	48 (4.6%)	187 (17.9%)
B. Distribution of mismatches by codon position[b]										
First base codon position	8.7%	5.4%	12.5%	8.6%	3.7%	13.1%	5.9%	11.1%	3.9%	10.5%
Second base codon position	0.0%	0.0%	3.7%	1.4%	0.0%	3.6%	2.9%	3.6%	2.0%	4.1%
Third codon position	91.3%	94.6%	83.8%	90.0%	96.3%	83.3%	91.2%	85.5%	94.1%	85.4%

[a] Number of mismatches/ORF length.

[b] Number of mismatches/total number of mismatches in ORF.

with the other serotypes consistently produce a less biased distribution of nucleotide substitutions to the third codon position (<86%) and consequently a greater likelihood that a nucleotide substitution will result in a change in the amino acid sequence. The frequency of third codon position mismatches has been shown to be an indicator of relatedness for reovirus (Weiner and Joklik, 1987; 1989). The more closely related two serotypes are, the greater the frequency (but not necessarily the actual number) of third codon position mismatches. A similar pattern is present in BTV; BTV-2, 10, 11, and 17 are very closely related, while BTV-13 is the most distant.

A pattern of relatedness among the BTV serotypes has emerged where BTV-13 has diverged extensively from the other 4 serotypes. This is seen on the level of divergence of the nucleotide (S1) and amino acid (VP7) sequence, conservation of ORFs, and distribution of nucleotide mismatches by codon usage. However, the relationships among BTV-2, 10, 11, and 17 are not so clear. Nucleic acid analyses suggest that BTV-2 is the most distantly related of this group and BTV-10 and 17 appear the closest. In order to clarify the relationship of these serotypes, transition frequencies and evolutionary distances (K values) were examined (Table 5; Kimura, 1980). Transition frequencies and evolutionary distances show that BTV-2 and BTV-13 are the most distantly related. Only 38.1% of their nucleotide substitutions represent transitions and they

TABLE 5

Comparison of Transition Frequencies and K Values from Segment S1 of the Five U.S. BTV Serotypes

	K value[a]				
	BTV-2	10	11	13	17
Transition frequency[b]					
BTV-2		0.063	0.068	0.288	0.065
10	78.3%		0.054	0.241	0.031
11	81.1%	85.7%		0.251	0.047
13	38.1%	56.4%	58.4%		0.243
17	78.9%	91.4%	90.4%	57.0%	

[a] Kimura, 1980.

[b] Number of transitions/total nucleotide differences x 100%.

possess a relatively large K value of 0.288. BTV-11 is the most closely related to BTV-13, but still has a low transition frequency of 58.4% and a K value of 0.241. This is in sharp contrast with the relationships among the other 4 serotypes where transition frequencies are greater than 78% and K values are approximately 1/4 that of comparisons with BTV-13. These analyses show that BTV-10 and 17 are the most closely related with a transition frequency of 90.4% and a K value of 0.031. Likewise, K values were calculated from the sequence of segment L2, which encodes the neutralization antigen, VP2 (Ghiasi et al., 1987). BTV-10:11 has a K value of 0.433 and BTV-10:17 has a K value of 0.410. Even though these values are higher than S1, which is the result of L2 being less conserved than S1, the same pattern of relatedness is observed with both the S1 and L2 segments. The BTV-10:17 relatedness is followed closely by BTV-11:17, BTV-10:11, BTV-2:10, BTV-2:17, and finally, BTV-2:11. The basic pattern of transition frequencies described here is similar to that observed with reovirus serotypes where the closely related serotypes 1 and 3 have a transition frequency of 82.5% and the much more distantly related serotypes pairs of 2:3, and 1:2 have a transition frequency of 52.5% for each pairing (Weiner and Joklik, 1989).

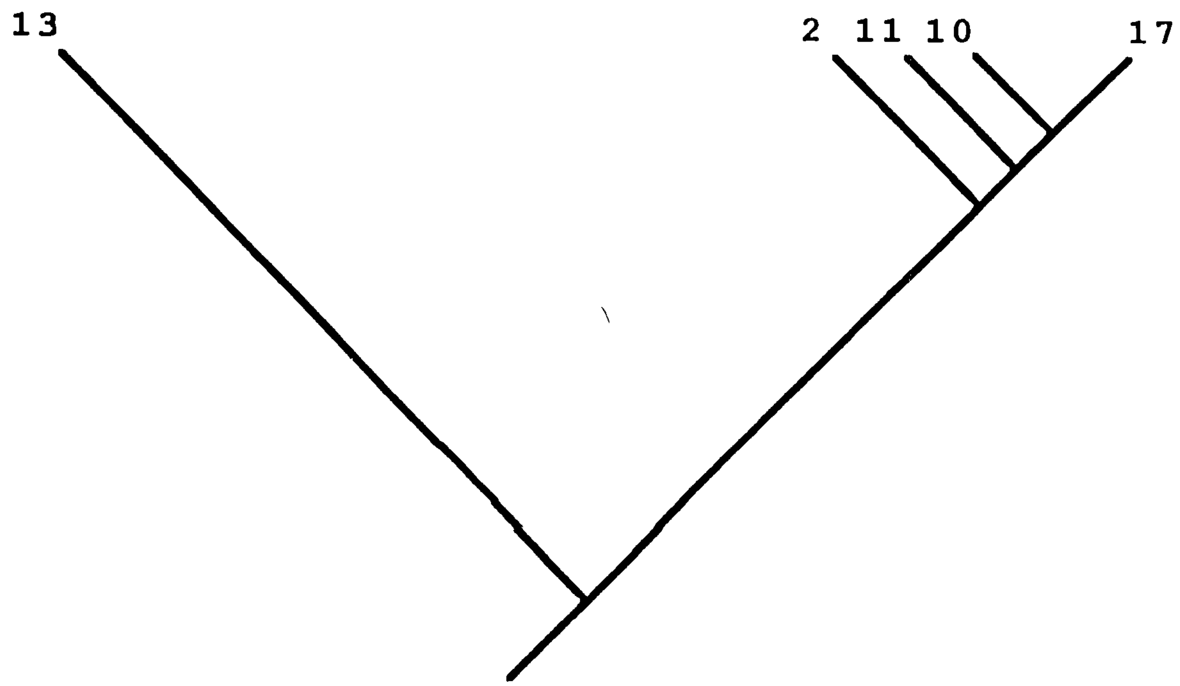
The temporal appearance of these virus isolates indicate that United States prototype BTV-10 was obtained in California in 1953; prototype BTV-11 was isolated in

Texas in 1962; prototype BTV-17 was isolated in Wyoming also in 1962; prototype BTV-13 was isolated in Idaho in 1967; and prototype BTV-2 (Ona-B) was isolated in Florida in 1982 (Sugiyama et al., 1982; Gibbs et al., 1983; Collisson et al., 1985). The close relationship of BTV-10, 11, and 17 indicate that they evolved from a relatively recent precursor, and the isolation data suggest that BTV-10 may be the ancestral serotype. However, retrospective serotyping of samples from epizootic outbreaks show that the BTV-11 strain used in this study may not have been the original BTV-11 strain isolated in the United States. It has also been suggested that as many as three BTV serotypes may have existed in 1953 (Barber, 1979). Therefore, care must be taken when using this historical information. Since BTV-17 is found only in the United States, it is assumed to have evolved here. This assumption, along with RNA fingerprinting data, was used to conclude that BTV-17 evolved from BTV-11 by genetic drift (Rao et al., 1983). Subsequent analysis by RNA/RNA hybridization provided no data to discredit this hypothesis (Squire et al., 1986; Kowalik and Li, 1987; Li et al., 1987). However, the analyses presented here overwhelmingly support the notion that segment S1 of BTV-17 evolved from BTV-10 and not BTV-11. The reason for this discrepancy is probably twofold. First, the RNA/RNA hybridization experiments, as performed, would not differentiate the subtle nucleotide differences between the S1 segments of

BTV-10, 11, and 17. Second, Rao et al. (1983) concluded that the RNA fingerprints of BTV-17 were comparable to BTV-11 but not to BTV-10 or BTV-13. The nucleotide sequence data of segment S1 (Yu et al., 1988; Kowalik and Li, 1989, Kowalik et al., 1990a; 1990b) and the alignment presented here (Fig. 2) support the absence of comparable RNA fingerprinting data between BTV-17 and 13, but clearly show that a high degree of similarity should have been observed between BTV-10 and 17 as well as between BTV-11 and 17. The RNA/RNA hybridization data as well as cDNA/RNA hybridization results show that, unlike the RNA fingerprinting data, segment S1 of BTV-10 is very closely related to both BTV-11 and 17 (Squire et al., 1986; Huismans and Cloete, 1987; Kowalik and Li, 1987). There is, however, evidence that at least one segment of BTV-17 evolved directly from BTV-11. Segment M3, which encodes the outer capsid protein VP5, has been shown by analyses of both the nucleic acids and proteins, to be highly variable (Squire et al., 1986; Huismans and Cloete, 1987; Kowalik and Li, 1987; Mecham et al., 1986; Mertens et al., 1987; Yang et al., 1990). These data show that segment M3 and VP5 of BTV-11 and 17 are similar, whereas they are unique among the other serotypes. It is concluded that BTV-17 evolved by a combination of genetic drift (segment S1 derived from BTV-10) and genomic reassortment (segment M3 from BTV-11).

Fig. 4 is a phylogenetic tree of the five United States

Figure 4. Phylogenetic tree of the five BTV serotypes based on the nucleotide sequence of segment S1. The tree was derived from the K values shown in Table 5 using the method of Fitch and Margoliash (1967). Relatedness between the serotypes is measured by the distance between the branch points of the tree.


13
2
11
10
17

BTV serotypes produced using a distance matrix method (Fitch and Margoliash, 1967) on the segment S1-derived data presented in Table 5. The tree is rooted; the five serotypes evolved from a common origin. BTV-13 has diverged from the other four serotypes into a separate gene pool. Since BTV-2 shared a recent common precursor with BTV-10, 11, and 17, a crude time frame can be estimated for the tree. Since prototype BTV-10 and BTV-2 have been separated for at least 29 years, it can be estimated from Fig. 4 that BTV-13 diverged away from the BTV-2, 10, 11, and 17 gene pool at least 90 years ago. By using either the 29 year gap between BTV-2 and BTV-10 or the 90 year gap between BTV-2 and 13, an evolutionary rate of approximately 2.2×10^{-3} nucleotide substitutions per site per year is predicted. This estimate is similar to the evolutionary rate of 5.7×10^{-3} substitutions/site/year calculated for the PB2 gene (RNA polymerase gene) of influenza (Schreier et al., 1988) and the slowest evolving gene (gag) of retroviruses (Gojiobori and Yokoyama, 1985; Hahn et al., 1986). If accurate, this would imply that there is no proofreading activity for the BTV RNA polymerase. This is not totally unexpected since there are no polymerase proofreading functions known in ssRNA viruses (Girones and Miller, 1989).

We have shown the application of the ClampR procedure to clone segment S1 from the five United States prototype BTV serotypes. The resulting clones were used in the

sequence analyses of S1 and VP7. Analyses of the evolutionary relatedness of the five serotypes by several criteria yielded the same pattern of relatedness; that the five United States BTV serotypes are derived from two distinct gene pools. The data were also used to make the first crude estimate of a mutation rate for a member of Reoviridae. The relative ease of the cloning approach and analyses described here should be applicable to further studies of the worldwide distribution and phylogeny of BTV and other RNA viruses.

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CHAPTER VI

SUMMARY

Bluetongue is the causative agent of bluetongue disease, a degenerative and, at times, fatal disease of ruminants. It has a worldwide distribution and is represented by 24 serotypes. It is of economic importance in endemic areas such as the United States because of exportation limitations on ruminants and their reproductive products. In order to gain a better understanding of the diversity of bluetongue virus, a study of the genetic relationships among the five bluetongue virus serotypes (BTV-2, 10, 11, 13, and 17) was undertaken.

The best measure of genetic relatedness entails sequencing of all or part of a genome of the entities in question. Since entire genome sequencing of five BTV isolates is not feasible, a marker sequence representative of each genome was chosen. Segment S1 was shown to be the best marker by its (1) variability, (2) its gene product, VP7 is not under immunoselection, and (3) expression of S1 is required for a virus isolate to be identified as a BTV.

Sequence data was obtained in two manners, both of which had to be developed during this investigation. When initial attempts to produce full-length clones were unsuccessful, a partial clone of segment S1 from BTV-13 was sequenced and primers based on this data were used to sequence rest of the gene directly. This required the

development of a direct dsRNA sequencing method. During this period attempts were also made to obtain full clones by first producing cDNA from genomic dsRNA with reverse transcriptase, then selectively amplifying full-length transcripts with the polymerase chain reaction (PCR) and cloning into plasmid vectors. This was theoretically possible because members of Reoviridae have conserved terminal sequences in individual dsRNA segments. By combining primers specific for the termini of segment S1 of BTV-10 with the previously synthesized cDNA, it was believed that full-length cDNA would be amplified by PCR and readily ligated into plasmids. At first this was unsuccessful. Success of this approach required optimization of each component of the reaction and the combination of reverse transcription and PCR in a one-tube, uninterrupted reaction (ClampR). Once full-length clones were made, sequencing of the cDNA was straight-forward.

Sequencing of S1 segments showed that they were well conserved among four of the five serotypes with BTV-10 and 17 being the closest and BTV-13 the most distantly related of the United States serotypes. The use of several different analyses produced the same result. A similar comparison of segment L2 confirmed this pattern. Analyses of the evolutionary distances by several different criteria all produced the same phylogenetic tree. By continually producing the same results, these different analyses

increase the confidence level in the accuracy of the proposed pattern of relatedness. This is important because some of the conclusions made in this study contradict earlier hypotheses. For the reasons discussed in Chapter V and the confirmation of the results obtain by segment S1 with segment L2, the pattern of genetic relatedness present in this study is the correct pattern.

In addition to their academic significance, the relationships found here have practical implications. The close relationships of BTV-2, 10, 11, and 17 suggest that they are all derived from a common source. This is further substantiated by the fact that BTV-2 and 10 have been separated by at least 29 years and still closely related. In addition, BTV-13 appeared in the United States 15 years before BTV-2 but is very different from these other serotypes. Taken together, this information supports the hypothesis made in this study that BTV-13 came from a different source. Therefore, BTV found in the United States is derived from (at least) two different gene pools. This conclusion will have significance in attempts to identify the sources of new BTV introductions into the United States. This will also serve as model for monitoring the spread of different BTV serotypes throughout the world.

In addition to the evolutionary relatedness of the BTV serotypes, two other observations/predictions were made

based on the nucleotide and deduced amino acid sequences of the five S1 segments. It is suggested that the 3' termini of the plus strands of the S1 segments all have the potential to form stable stem-loop structures. I believe that the actual structures are much more complicated than those predicted here, but confirmation of this will require indepth structural studies. It was also observed that secondary structures in the 3' termini appear to be common features of both ssRNA and dsRNA RNA viruses. Very little is known on the biological significance of these observations. However, it is suggested that they could potentially play a role in the undiscovered genome segment sorting mechanism that is required for the proper segregation of each gene segment during virus maturation.

Cores of ssRNA viruses all have eight-stranded antiparallel ß-barrels as their main structural component. Secondary structure analyses predict a similar motif for VP7. This protein is the major core protein of BTV so this not too unexpected. Although no actual structure determination of a dsRNA virus has been made, the putative ß-barrel and its analogy with other proteins with similar function and known structure, allows for further predictions. First, the conservation of this structural element in ssRNA viruses is used as evidence for a common origin of ssRNA viruses. The prediction (and future determination) of a similar motif in dsRNA viruses would

support the hypothesis that dsRNA viruses also evolved from this precursor virus. Second, the ß-barrel is a target of several drugs used to eradicate picornavirus infections. The predicted ß-barrel in BTV could serve as a similar target. This would be the first development of drugs directed against an orbivirus that I am aware of.

The methodology and approaches developed here have greatly reduced the time, effort, and expense to do cloning and nucleic acid sequencing studies of dsRNA viruses. This will permit rapid accumulation of data on the evolutionary relatedness of many BTV isolates as well as other members of *Orbivirus* and the Reoviridae family. The ability to rapidly produce full-length clones and to modify the termini as needed will greatly facilitate gene expression studies useful in deciferring the roles of individual proteins and in determining the entire BTV replication cycle.

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RESEARCH EXPERIENCE

1984-present: Characterization of the molecular evolution of segmented double-stranded RNA viruses using an Orbivirus, bluetongue virus, as a model. Studies include cognate gene identification, RNA/RNA hybridization, cloning of the dsRNA genome, DNA and RNA sequencing, and computer analyses.

1983-1984: Studies on nitrogen fixation of associative symbionts from Achillea millefolium and Hackelia microntha in axenic and gnotobiotic growth.

1982-1983: Characterization of the diether phytanyl phosphatidylglycerolphosphate moiety of Halobacterium membranes for potential use as an emulsifying agent.

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1989: Travel Fellowship, American Society for Virology

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ABSTRACTS:

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