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Complete Sequence of Bluetongue Virus L2 RNA That Codes for the Antigen Recognized by Neutralizing Antibodies

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The complete sequence of the RNA which encodes the major outer-shell-neutralizing antigen (VP2) of bluetongue virus serotype 10 was determined from overlapping cDNA clones inserted into pBR322. The segment L2 RNA was 2,926 base pairs long (1.87×10^6 daltons) and had, in one strand, an open reading frame capable of coding for a protein that had a calculated size of 111,122 daltons (956 amino acids) and a +11.5 net charge. The coding strands of both the L2 gene and the group-specific L3 gene of bluetongue virus serotype 17 (M. Purdy, J. Petre, and P. Roy, J. Virol. 51:754–759, 1984) had common sequences of some six nucleotides at their 5′ termini (namely, GUUAAA. . .) and eight nucleotides (namely, . . .ACACUUAC) at their 3′ termini. Both had short 5′ noncoding regions with AUG codons at residues 20 to 22 (L2) and 18 to 20 (L3). The sequences flanking these AUG codons were similar (A/GCCAUGG). The 3′ noncoding regions were longer (36 nucleotides for L2, 49 nucleotides for L3). The predicted amino acid sequence of the L2, compared with the similarly sized L3 gene product, was rich in cysteine residues and charged amino acids.

Bluetongue virus (BTV), a member of the *Orbivirus* genus, family *Reoviridae*, has a genome consisting of 10 segments of double-stranded RNA (dsRNA). The virus, which is transmitted by *Culicoides* species, is responsible for disease in ruminants, primarily sheep. In the virus particle, the dsRNA is surrounded by a double capsid shell, the outer capsid consisting of two polypeptides (VP2 and VP5) and the inner core consisting of two major (VP3 and VP7) and three minor (VP1, VP4, and VP6) polypeptides (9, 15). Huismans and Erasmus (3) have shown that VP2 is the serotype-specific antigen by using immunoprecipitation techniques. They subsequently demonstrated that the solubilized VP2 polypeptide is recognized by neutralizing antibodies in rabbits and sheep and can protect sheep against viral infection. They also found that VP2 is required for the hemagglutination of sheep erythrocytes as well as for cellular adsorption (3, 4). It has been demonstrated both in vivo (with intertypic reassortant viruses; 5) and in vitro (by a translation system; 13) that BTV RNA segment 2 (i.e., the L2 RNA) codes for VP2. In this study, we describe the cloning of the L2 gene of BTV serotype 10 (BTV-10) into pBR322 and present the complete sequence of the cloned L2 gene and its product, the VP2 antigen.

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The procedure used to obtain the complete sequence of the L2 RNA species involved the synthesis of cDNA followed by cloning and sequencing of the derived DNA. The L2 RNA species was polyadenylated at its 3′ ends, and cDNA copies synthesized with reverse transcriptase, deoxyribonucleoside triphosphates, and an oligo$(dT)_{12-18}$ primer as described previously (11, 14). The RNA template-cDNA product duplexes were then tailed with deoxycytidylic acid and cloned into the *Pst*I site of pBR322 (8). Clones representing the L2 DNA were identified by colony hybridization with a short-copy cDNA probe prepared with polyadenylated template RNA and an oligo$(dT)_{12-18}$ primer (2). For confirmation, BTV-10 RNA segments separated on agarose gels were blotted in Genescreen paper and hybridized to nick-translated DNA representing the positive clones (11). The clones annealed specifically to the L2 RNA of BTV-10. Several L2 clones were identified, *Hin*fI restriction patterns of each clone were compared as described previously (1), and the two longest clones (2.86 and P1-79) were sequenced (Fig. 1 and 2). Sequencing was carried out on end-labeled, strand-separated restriction fragments of plasmid DNA containing viral DNA inserts by the method of Maxam and Gilbert (10). A *Dde*I restriction fragment from clone P1-79 (representing residues 727 to 834) and a *Pst*I restriction fragment from clone 2.86 (representing residues

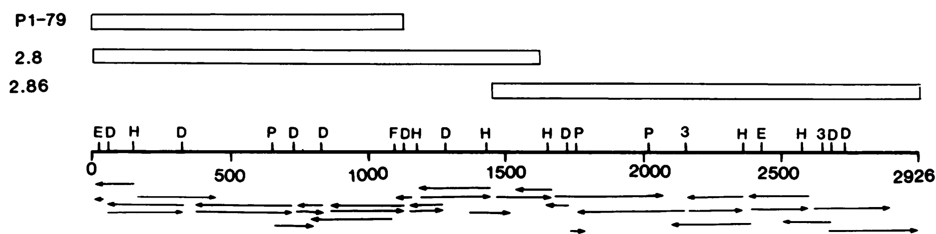

FIG. 1. Sequence strategy used to determine the sequence of the cDNA clones of BTV-10 L2 gene. The restriction fragments of the three clones (P1-79, 2.8, and 2.86) used to obtain the DNA sequence are shown by arrows representing the individual strands that were sequenced. Restriction site symbols: H, *Hin*fI; D, *Dde*I; 3, *Hin*dIII; F, *Fnu*4HI; P, *Pvu*II; E, *Eco*RI.

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               M  E  E  F  V  I  P  V  F  S  E  R  D  I  P  Y  S  L  L  N  H  Y  P  L  A  I  Q  I  D  V  K  V  D  D
GTTAAAAGAGTGTTCTACCATGGAGGAATTCGTCATACCAGTGTTTTCTGAGAGAGATATTCCATATTCACTGCTAAATCATTACCCACTAGCTATACAGATAGATGTTAAAGTTGATGA
        10        20        30        40        50        60        70        80        90       100       110       120

 E  G  G  K  H  N  L  I  K  I  P  E  S  D  M  I  D  V  P  R  L  S  I  I  E  A  L  N  Y  R  P  K  R  N  D  G  V  V  V  P
TGAAGGTGGGAAACATAATCTTATCAAGATACCTGAATCGGATATGATCGACGTACCGAGGTTAAGTATCATAGAGGCTCTAAATTATAGACCGAAAAGGAACGATGGTGTGGTTGTGCC
       130       140       150       160       170       180       190       200       210       220       230       240

 R  L  L  D  I  T  L  R  A  Y  D  N  R  K  S  A  K  N  A  K  G  V  E  F  M  T  D  T  K  W  M  K  W  A  I  D  D  K  M  D
GAGATTGCTAGATATAACGCTACGCGCTTATGATAATAGGAAATCCGCAAAGAACGCGAAAGGAGTTGAATTTATGACAGATACTAAGTGGATGAAATGGGCAATTGACGACAAAATGGA
       250       260       270       280       290       300       310       320       330       340       350       360

 I  Q  P  L  K  V  T  L  D  N  H  C  S  V  N  H  Q  L  F  N  C  I  V  K  A  R  S  A  N  A  D  T  I  Y  Y  D  Y  Y  P  L
TATACAACCCCTTAAGGTTACTCTGGATAACCACTGTTCCGTTAACCATCAACTCTTCAATTGTATCGTTAAGGCGAGGTCAGCAAATGCAGACACTATTTATTACGACTATTACCCATT
       370       380       390       400       410       420       430       440       450       460       470       480

 E  N  G  A  K  R  C  N  H  T  N  L  D  L  L  R  S  L  T  T  T  E  M  F  H  I  L  Q  G  A  A  Y  A  L  K  T  T  Y  E  L
GGAAAATGGAGCGAAAAGATGTAATCATACGAACCTGGATTTATTACGAAGTTTAACAACGACGGAGATGTTCCACATCTTACAGGGAGCGGCTTACGCCTTAAAAACAACGTATGAATT
       490       500       510       520       530       540       550       560       570       580       590       600

 V  A  H  S  E  R  E  N  M  S  E  S  Y  Q  V  G  T  Q  R  W  I  Q  L  R  K  G  T  K  I  G  Y  R  G  Q  P  Y  E  R  F  I
AGTAGCTCATTCCGAAAGAGAAAACATGAGCGAGAGTTATCAGGTTGGGACGCAGAGATGGATACAGCTGCGTAAAGGTACTAAAATTGGATATAGAGGGCAGCCGTACGAACGATTTAT
       610       620       630       640       650       660       670       680       690       700       710       720

 S  S  L  V  Q  V  I  I  K  G  K  I  P  D  E  I  R  T  E  I  A  E  L  N  R  I  K  D  E  W  K  N  A  A  Y  D  R  T  E  I
ATCCAGCTTAGTTCAAGTAATCATTAAAGGGAAAATTCCGGATGAGATTAGAACCGAAATCGCTGAACTTAATAGAATAAAAGATGAGTGGAAAAACGCCGCGTATGACAGAACTGAGAT
       730       740       750       760       770       780       790       800       810       820       830       840

 R  A  L  E  L  C  K  I  L  S  A  I  G  R  K  M  L  D  V  Q  E  E  P  K  D  E  M  A  L  S  T  R  F  Q  F  K  L  D  E  K
ACGTGCATTGGAGCTCTGCAAGATACTTTCGGCGATCGGACGTAAAATGTTAGATGTTCAAGAAGAGCCAAAGGATGAGATGGCGTTATCTACTAGATTTCAGTTCAAGTTAGACGAGAA
       850       860       870       880       890       900       910       920       930       940       950       960

 F  I  R  T  D  Q  E  H  V  N  I  F  K  V  G  G  S  A  T  D  D  G  R  F  Y  A  L  I  A  I  A  G  T  D  T  Q  Q  G  R  V
ATTCATTAGGACTGATCAAGAACACGTAAATATATTTAAAGTAGGTGGTTCCGCAACAGATGATGGGAGATTTTACGCGCTAATCGCTATAGCAGGTACCGATACGCAGCAAGGCCGTGT
       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080

 W  R  T  N  P  Y  P  C  L  R  G  A  L  I  A  A  E  C  E  L  G  D  V  Y  F  T  L  R  Q  T  Y  K  W  S  L  R  P  E  Y  G
TTGGAGAACGAACCCTTATCCATGTTTGCGTGGTGCGCTAATTGCTGCTGAGTGTGAGCTCGGCGATGTGTACTTCACATTGCGTCAAACGTATAAATGGAGTCTGCGTCCAGAATATGG
      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200

 Q  R  E  R  P  L  E  D  N  K  Y  V  F  A  R  L  N  L  F  D  T  N  L  A  V  G  D  E  I  I  H  W  R  Y  E  V  Y  Q  P  K
TCAGCGTGAGAGGCCGTTAGAAGACAATAAATATGTGTTCGCTCGTCTTAATCTATTTGATACTAACTTAGCCGTTGGGGATGAAATCATTCACTGGCGTTATGAGGTTTATCAGCCTAA
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320

 E  T  T  H  D  D  G  Y  I  C  V  S  Q  K  G  D  D  E  L  L  C  E  V  D  E  D  R  Y  K  E  M  F  D  R  M  I  Q  G  G  W
AGAAACAACTCATGATGATGGATATATCTGTGTGTCACAGAAAGGTGATGATGAATTATTATGTGAGGTTGATGAAGATAGGTATAAGGAGATGTTTGATCGTATGATTCAAGGCGGATG
      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440

 D  Q  E  R  F  K  L  H  N  I  L  T  E  P  N  L  L  T  I  D  F  E  K  D  A  Y  L  G  A  R  S  E  L  V  F  P  P  Y  Y  D
GGATCAGGAACGGTTCAAATTACACAACATATTGACTGAACCAAACCTACTTACCATCGATTTCGAGAAGGATGCTTACCTTGGCGCGCGTTCTGAATTAGTATTCCCGCCCTATTATGA
      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560

 K  W  I  N  S  P  M  F  N  A  R  L  K  I  A  R  G  E  I  A  T  W  K  A  D  D  P  W  S  N  R  A  V  H  G  Y  I  K  T  S
CAAATGGATCAATTCACCGATGTTTAATGCCCGGCTTAAAATTGCGCGTGGCGAGATTGCAACCTGGAAGGCGGATGATCCTTGGAGTAATCGGGCGGTTCATGGGTATATAAAGACGTC
      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680

 A  E  S  L  E  Y  A  L  G  P  Y  Y  D  L  R  L  Q  L  F  G  D  T  L  S  L  G  Q  R  Q  S  A  V  F  E  H  M  A  Q  Q  D
AGCGGAATCTTTAGAGTATGCGTTAGGGCCATATTATGACCTACGTCTGCAGTTATTCGGCGACACCCTCAGTCTGGGACAAAGACAGTCAGCTGTATTCGAACATATGGCTCAACAAGA
      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800

 D  F  S  T  L  T  D  Y  T  K  G  R  T  V  C  P  H  S  G  G  T  F  Y  T  F  R  K  V  A  L  I  I  L  G  N  Y  E  R  L  D
TGATTTTTCTACGCTAACGGATTATACGAAAGGAAGAACAGTGTGCCCTCACTCAGGCGGGACTTTTTACACTTTCCGCAAGGTAGCGTTGATTATTTTATCGAATTACGAACGATTGGA
      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920

 P  S  L  H  E  G  R  E  H  E  T  Y  M  H  P  A  V  N  D  V  F  R  R  H  V  L  E  M  K  D  F  S  Q  L  I  C  F  V  F  D
CCCAAGTTTACATGAGGGGAGGGAACATGAGACATACATGCACCCGGCAGTTAATGATGTCTTTAGACGACACGTTTTAGAAATGAAAGATTTTTCCCAGCTGATTTGTTTTGTGTTCGA
      1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040

 Y  I  F  E  K  H  V  Q  L  R  N  A  K  E  A  R  R  I  I  Y  L  I  Q  N  T  S  G  A  Y  R  L  D  V  L  R  E  A  F  P  N
CTACATTTTTGAGAAACATGTACAGCTACGTAATGCCAAAGAGGCGAGAAGGATTATATATTTAATACAGAATACGTCCGGTGCGTATCGATTAGATGTTTTACGCGAAGCTTTCCCCAA
      2050      2060      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160

 F  L  K  H  V  M  N  L  R  D  V  K  R  I  C  D  L  N  V  I  N  F  F  P  L  L  F  L  V  Q  D  N  I  S  Y  W  H  R  Q  W
CTTTTTAAAGCACGTTATGAATTTACGTGATGTGAAACGTATATGCGATCTGAATGTGATTAATTTCTTTCCATTGTTATTTTTGGTTCAAGATAATATTTCATATTGGCATAGACAGTG
      2170      2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280

 S  I  P  M  I  L  F  D  Q  V  I  R  L  I  P  V  E  V  G  A  Y  A  N  R  F  G  L  K  S  F  F  N  F  I  R  F  H  P  G  D
GTCGATCCCAATGATTTTATTTGATCAAGTTATCAGATTAATTCCCGTCGAAGTCGGGGCATACGCAAATAGATTTGGACTTAAGAGCTTCTTCAATTTCATTAGGTTTCACCCCGGCGA
      2290      2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400

 S  K  K  R  Q  D  A  D  D  T  H  K  E  F  G  S  I  C  F  E  Y  Y  T  T  T  K  I  S  Q  G  E  I  D  V  P  V  V  T  S  K
TTCAAAGAAGAGACAGGATGCGGATGACACCCATAAAGAATTCGGCTCAATATGTTTCGAGTATTACACTACGACAAAGATCTCTCAAGGTGAGATAGATGTTCCGGTCGTTACGTCTAA
      2410      2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520

 L  D  T  L  K  L  H  V  A  S  L  C  A  G  L  A  D  S  L  V  Y  T  L  P  V  A  H  P  K  K  S  I  V  L  I  I  V  G  D  D
ATTAGATACACTTAAGTTGCACGTAGCTTCCTTATGCGCGGGGCTAGCTGACTCTCTAGTGTATACGCTGCCTGTAGCGCACCCGAAGAAAAGTATCGTTCTAATTATTGTGGGGGATGA
      2530      2540      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640

 K  L  E  P  Q  I  R  S  E  Q  I  V  N  K  Y  Y  Y  S  R  R  H  I  S  G  V  V  S  I  C  V  N  Q  G  G  Q  L  K  V  H  S
TAAGCTTGAACCACAAATACGCTCAGAGCAAATCGTAAATAAGTACTATTATTCACGGAGGCACATCTCAGGGGTTGTTTCGATCTGCGTGAACCAGGGTGGACAACTGAAGGTGCATTC
      2650      2660      2670      2680      2690      2700      2710      2720      2730      2740      2750      2760

 M  G  I  T  R  H  R  I  C  D  K  S  I  L  K  Y  K  C  K  V  V  L  V  R  M  P  G  H  V  F  G  N  D  E  L  M  T  K  L  L
CATGGGGATAACGAGACATCGGATATGTGATAAGTCGATATTGAAATATAAATGCAAAGTAGTATTAGTTAGGATGCCAGGACATGTCTTCGGAAATGATGAATTAATGACGAAGCTACT
      2770      2780      2790      2800      2810      2820      2830      2840      2850      2860      2870      2880

 N  V  *
AAATGTTTAGGTCCTGTGACATGGACCGGTAGCCCTCTTACACTTAC
      2890      2900      2910      2920
```

FIG. 2. Nucleotide sequence of the cloned L2 gene encoding the serotype-specific capsid protein from BTV-10. The open reading frame begins with ATG codon at position 20 to 22 and is terminated by the TAG at position 2888 to 2890. The largest predicted gene product is shown by the single-letter amino acid convention (Table 1). Residues are centered over the corresponding codons. Nucleotides are numbered below their respective residues.

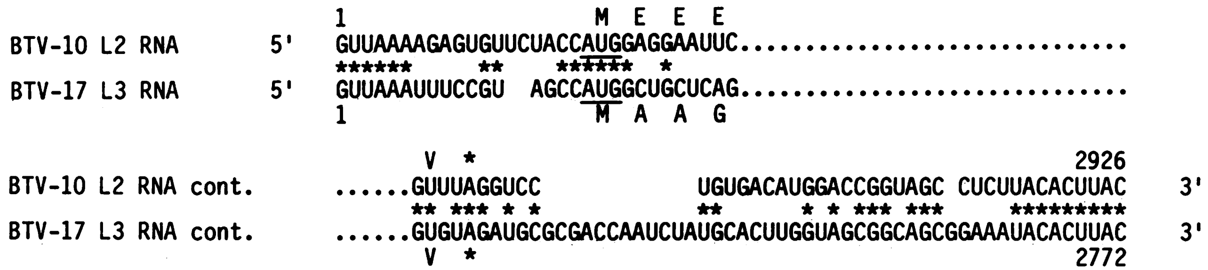

FIG. 3. Sequences of the 5′ and 3′ terminal regions of the L2 gene of BTV-10 and the L3 gene of BTV-17. The base homologies through the first AUG initiation codons (5′ proximal) and the termination of UAG codons (3′ proximal) are indicated by asterisks. The conventional single-letter amino acids are shown over the corresponding codons. The sequences are aligned on the basis of their conserved features.

1471 to 1727) were recovered, nick translated, and used as probes to identify additional L2 clones. Several clones with restriction fragments homologous to those of P1-79 and 2.86 were identified, and the largest clone (2.8) was sequenced (Fig. 1, 2). The sequencing data indicated that the L2 RNA segment was 2,926 base pairs in length (molecular weight, 1.87×10^6).

Previous studies involving sequence analyses of the viral RNA species of BTV strains have characterized the 3′-terminal sequences of the 10 RNA species (12). It has been deduced that the viral RNA species have conserved 5′ and 3′ terminal sequences (viz., 5′ GUUAAA. . ., and . . .UACACUUAC 3′). By comparison with these data, the sequence analyses of the L2 DNA clones indicated that both ends of the L2 RNA species were represented. When the RNA sequences of the coding strands of the L2 and the previously characterized L3 gene were compared (Fig. 3), it was observed that both had a conserved 5′ end sequence (GUUAAA. . .), a conserved 3′ end sequence (. . .UACACUUAC), short 5′ noncoding regions (L2, 19 nucleotides; L3, 17 nucleotides), longer 3′ noncoding regions (L2, 36 nucleotides; L3, 49 nucleotides), and a consensus sequence flanking the first AUG codon (viz., A/GCCAUGG). Overall, the nucleotide composition of the coding strand of the L2 gene was 31.0% A, 27.6% U, 23.7% G, and 17.7% C.

TABLE 1. Amino acid composition of two BTV gene products

Amino acid	No. of residues	
	BTV-10 L2	BTV-17 L3
Alanine (A)	53	65
Arginine (R)	65	69
Aspartate (D)	69	59
Asparagine (N)	41	42
Cysteine (C)	16	5
Glutamate (E)	60	50
Glutamine (Q)	37	42
Glycine (G)	49	42
Histidine (H)	29	16
Isoleucine (I)	70	65
Leucine (L)	90	82
Lysine (K)	61	28
Methionine (M)	20	37
Phenylalanine (F)	43	39
Proline (P)	36	46
Serine (S)	47	41
Threonine (T)	48	51
Tryptophan (W)	13	10
Tyrosine (Y)	47	39
Valine (V)	62	73
Net charge	+11.5	−4
Size	111,112 daltons	103,416 daltons

Analysis of the deduced viral L2 RNA sequences identified only one long open reading frame (Fig. 2). The first AUG condon was at residues 20 to 22; it initiated an open reading frame that concluded with a UAG termination codon at residues 2888 to 2890. The encoded gene product was 956 amino acids in length with a calculated size of 111,122 daltons (Table 1). Within the first 400 nucleotides, additional AUG codons in the same reading frame were present at residues 164 to 166, 314 to 316, 332 to 334, and 356 to 358. In other reading frames, AUG codons were present at residues 105 to 107, 117 to 119, 120 to 122, 225 to 227, and 270 to 272. The first three of these were in the same reading frame and could potentially code for the peptide MLKLMMKVGNIILSRYLNRI. Whether this peptide is made in virus-infected cells is not known.

The L3 gene product of BTV-17 had a low content of lysine in comparison to the L2 gene product. In part because of this, but also because of other amino acids, the L2 gene product had more charged amino acids than does the L3 product and overall was more hydrophilic than the L3 gene product (Fig. 4). In addition, it contained three times more cysteine amino acids (16 residues as opposed to 5 residues) (Table 1). Overall, the L2 gene product had a net +11.5 charge; the net charge of the L3 gene product was −4, assuming that lysine and arginine each had a +1 charge, that histidine had a charge of +1/2, and that aspartic and glutamic acids each provided a −1 charge at a neutral pH (Table 1).

The strategy we used for cloning the dsRNA segments of BTV involved RNA-cDNA hybrids instead of dsDNA. This method made the cloning procedures much simpler than that used previously for dsRNA viruses. Overlapping clones representing the complete L2 gene of BTV-10 were obtained by this method. The nucleotide sequence of L2 gene was 2,926 bases long, in good agreement with the length of 2.9 kilobases length calculated by gel electrophoresis (15). Clone 2.8 represented the 5′ terminus of BTV-10 RNA segment L2 (Fig. 1), and clone 2.86 represented the 3′ terminus. They had an overlapping region of 250 base pairs. Analysis of the terminal sequences showed that they were identical to those deduced by direct RNA sequence analyses for the termini of BTV-10 segment L2 genomic RNA (12).

The DNA sequence of the L2 gene had a single long open reading frame, beginning with an AUG codon at residues 20 to 22 and terminating with a UAG codon at residues 2888 to 2890. This reading frame coded for a protein of 956 amino

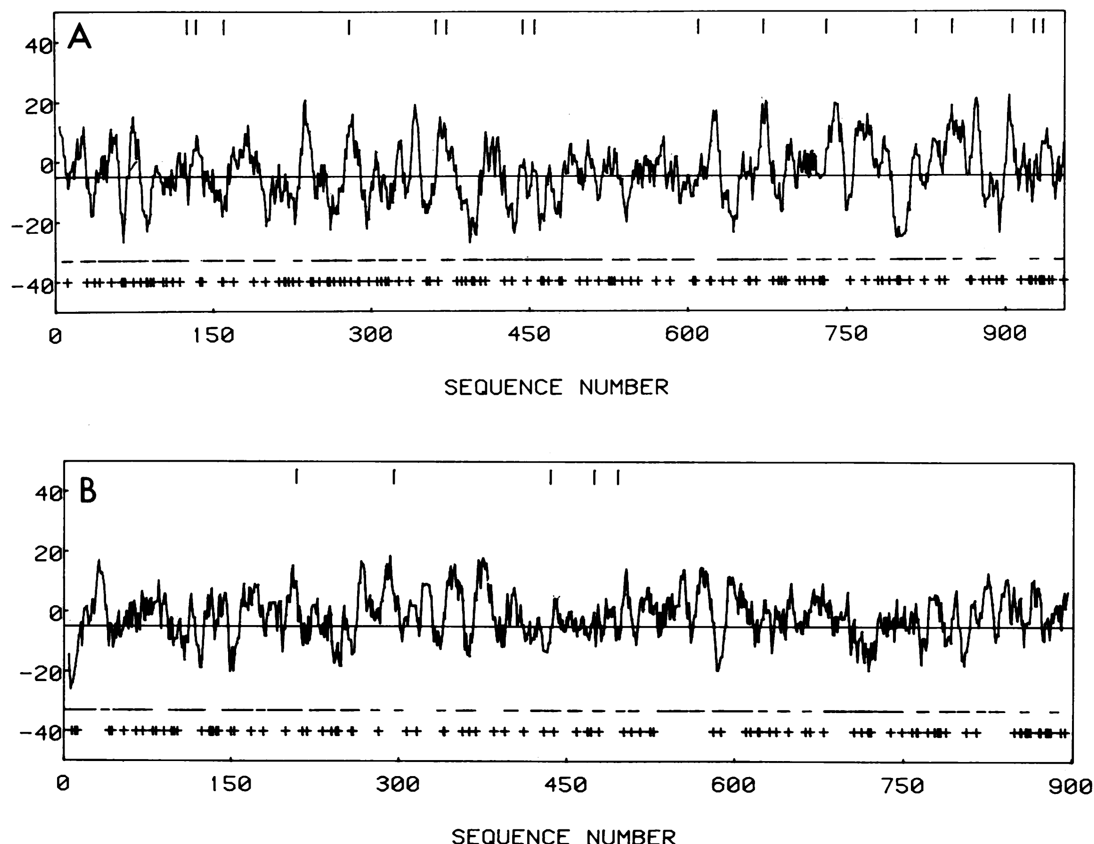

FIG. 4. Hydropathic plot and cysteine and charged amino acid distributions for the predicted RNA L2 gene product of BTV-10, representing the outer-capsid-neutralizing antigen (A) and for the RNA L3 gene product of BTV-17, representing a group-specific core protein (B). The regions of the predicted proteins with a net hydrophobicity (areas above the center line) or hydrophilicity (areas below the center line) are displayed (7), as well as the distribution of charged amino acids (R, K, D, and E) and cysteine residues (vertical bars). Both plots involve a span setting of nine amino acids.

acids. The first AUG codon is the most likely initiation point for the L2 gene product. When compared with the deduced sequence for the L3 RNA (11), the AUG codons of the two RNA species had a consensus flanking sequence of A/GCCAUGG, which is comparable to the consensus flanking sequence identified by Kozak (6). The 3′ termini of the two genes exhibited conserved sequences (Fig. 3). Presumably they are important in the initiation of negative-sense RNA intermediates of replication. The reading frame that generated the 956-amino-acid protein is assumed to be the correct reading frame, as all other reading frames failed to generate any amino acid sequence of significant length.

Since the L2 gene encodes for the neutralizing antigen that resides in the outside capsid of the virus particles (5, 13), it may be expected to have a hydrophilic nature, particularly the part that is exposed on the outer surface. Analysis of the L2 gene product for the regions of hydrophobicity (7) did not identify any strong hydrophobic sequence (Fig. 4). Overall, though, and in comparison to the hydropathic plot for the BTV L3 gene product, the L2 protein had a more hydrophilic character and many more charged amino acids (i.e., including histidines, 284 amino acids as opposed to 222). It also had three times more cysteine residues.

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