J. gen. Virol. (1987), **68**, 2967–2973. *Printed in Great Britain*

Key words: *bluetongue virus/neutralization protein/antigenic variation*

Variation in the Bluetongue Virus Neutralization Protein VP2

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(*Accepted 16 July 1987*)

SUMMARY

To determine the extent and nature of the antigenic variation of four U.S.A. serotypes of bluetongue virus (BTV), the complete nucleotide sequence was determined for cDNA clones representing the L2 dsRNA of BTV serotype 13, the gene that codes for the outer capsid neutralization antigen (VP2). The predicted amino acid sequence of the protein was compared with the VP2 sequences of the U.S.A. serotypes of BTV-10, BTV-11 and BTV-17. Diagon comparisons, hydropathic plots and analyses of potential secondary structure of the four proteins indicated that all four VP2 proteins were structurally similar. However, the VP2 protein of BTV-13 was found to exhibit only 40% homology with the VP2 species of the other three viruses. The comparative sequence data indicated that there were regions of the proteins with greater variability than other regions, as expected for proteins that vary antigenically but are structurally similar.

Bluetongue virus (BTV) is the causative agent of bluetongue disease in domestic ruminants (sheep, cattle). It is a member of the *Orbivirus* genus (Reoviridae family). Twenty-four distinct serotypes of the virus have been recognized on the basis of serum neutralization tests. The serotype diversity of BTV is of veterinary importance particularly in the U.S.A. and southern Europe, even though only a few virus serotypes have been identified in these locations. The BTV particle is composed of ten dsRNA segments surrounded by an icosahedral, double capsid shell. The outer capsid consists of two polypeptides, one of which (VP2) is a major serotype-specific antigen (Kahlon *et al.*, 1983; Huismans & Erasmus, 1981). Solubilized VP2 protein induces neutralizing antibodies that protect sheep against infection by BTV (Huismans *et al.*, 1983). We have previously shown that the viral L2 dsRNA segment encodes the VP2 protein and that it is one of the most variable segments (Rao *et al.*, 1983*a*; Sugiyama *et al.*, 1981) among the different BTV serotypes in the U.S.A. A study of the serologically closely related U.S.A. viruses BTV-10, -11 and -17 has shown that their L2 genes and VP2 proteins are very similar in sequence (Ghiasi *et al.*, 1987). The fourth U.S.A. serotype, BTV-13, is serologically more distinct. Prior genetic and molecular studies (Sugiyama *et al.*, 1982; Roy *et al.*, 1985) suggested that in the U.S.A. BTV-13 may have evolved differently. In order to investigate the relationship of BTV-13 to the other virus serotypes, the complete nucleotide sequence of the L2 gene of BTV-13 has been determined and compared with those of BTV-10, BTV-11 and BTV-17 (Purdy *et al.*, 1985; Ghiasi *et al.*, 1987). The results provide new insights into the extent and nature of genetic variation in the gene coding for the antigenic coat protein of these viruses.

The procedure used to obtain the complete nucleotide sequence of BTV-13 L2 RNA species involved the synthesis of cDNA followed by cloning and sequencing of the derived L2-specific plasmid DNA. In brief, the viral L2 RNA species were polyadenylated at their 3′ ends and cDNA copies of both strands were synthesized using reverse transcriptase, deoxyribonucleoside triphosphates and an oligo(dT) primer (Purdy *et al.*, 1984). After removal of the RNA templates by hydrolysis with KOH, the cDNA products were self-annealed and, to ensure that the

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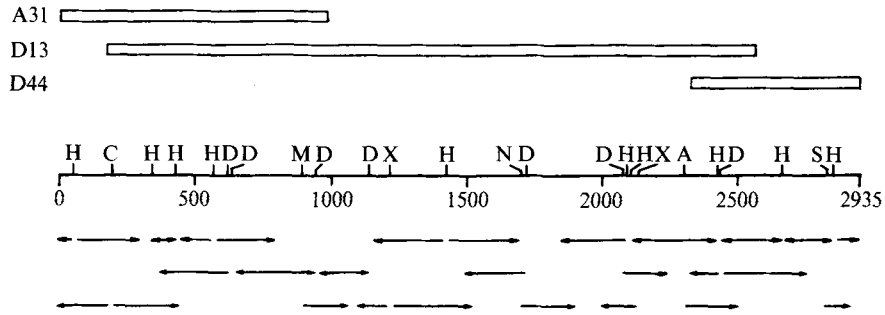

Fig. 1. Sequence strategy used to determine the sequence of the cDNA clones of BTV-13 L2 gene. The distance and directions in which individual strands of three overlapping clones were sequenced are shown by the solid arrows. Restriction site symbols used are as follows: A, *Ava*II; C, *Cla*II; D, *Dde*I; H, *Hin*fI; M, *Mlu*I; N, *Nde*I; S, *Sau*3AI; X, *Xho*I.

products were full-length, their 3′ ends were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase I. The DNA species were then tailed with dC and cloned into the *Pst*I site of pBR322. Approximately 100 clones representing the L2 gene were identified by colony hybridization using a short-copy cDNA probe which was transcribed from polyadenylated viral RNA using an oligo(dT) primer. The plasmid DNA prepared from 50 colonies was digested with *Hin*fI and the sizes of the inserted DNA were determined by electrophoresis of the products in 4% polyacrylamide gels. The restriction patterns of the recombinants showed that ten of the clones possessed viral DNA inserts that were between 500 and 2000 bp in length. In order to confirm that they represented the L2 segment, BTV-13 RNA segments were resolved by agarose gel electrophoresis, blotted on Genescreen paper (New England Nuclear) and hybridized to nick-translated DNA representing the ten positive clones. All the clones annealed specifically to the L2 RNA of BTV-13 (data not shown).

The sequence of the BTV-13 L2 gene was determined (Maxam & Gilbert, 1980) on strand-separated, end-labelled, restriction DNA samples from three overlapping clones (A31, D13 and D44) using the restriction endonuclease fragments shown in Fig. 1. The complete nucleotide sequence of the cDNA, in the positive (mRNA) sense, is presented in Fig. 2 with the predicted amino acid sequence of the single long open reading frame shown above it. The L2 RNA of BTV-13 was deduced from these analyses to be 2935 nucleotides long, larger than the previously reported L2 genes of BTV-10, -11 and -17 (i.e. BTV-10 and BTV-11, 2926 nucleotides; BTV-17, 2923 nucleotides). The BTV-10, -11 and -17 L2 RNA species contain a short 5′ non-coding region of 19 nucleotides and a longer 3′ non-coding region of 36 nucleotides (excluding the stop codon). As shown in Fig. 2 the BTV-13 L2 5′ non-coding region was longer (21 nucleotides), and exhibited greater sequence divergence by comparison with the other three L2 RNA species, although the six terminal 5′ nucleotides were identical (i.e. 5′ GUUAAA...; Rao *et al.*, 1983*b*). By contrast, the 3′ non-coding regions of all four viral L2 RNA species are more conserved (60 to 61%).

The coding strand of the BTV-13 L2 RNA had a calculated base composition of 27·7% U, 30·2% A, 18·1% C and 24% G (similar to the base compositions of the coding strands of the other three BTV L2 RNA species). There was only one long open reading frame in the BTV-13 L2 RNA. It coded for a primary gene product of 959 amino acids with an estimated M_r of 112565. The calculated net positive charge of the protein at pH 7·0 was +4·5, assuming that glutamic and aspartic acids each have charges of −1, arginine and lysine +1, and histidine +0·5 at neutral pH. The striking feature of this protein was that its net charge was much lower than those of the L2 gene products of the other three BTV serotypes (Purdy *et al.*, 1985; Ghiasi *et al.*, 1987).

Alignments of the VP2 sequences of BTV-10, -11, -13 and -17 are shown in Fig. 3. The close similarities of the VP2 sequences of the U.S.A BTV-10, -11 and -17 serotypes are indicated by the fact that only one gap was required for maximum homologous alignment between them

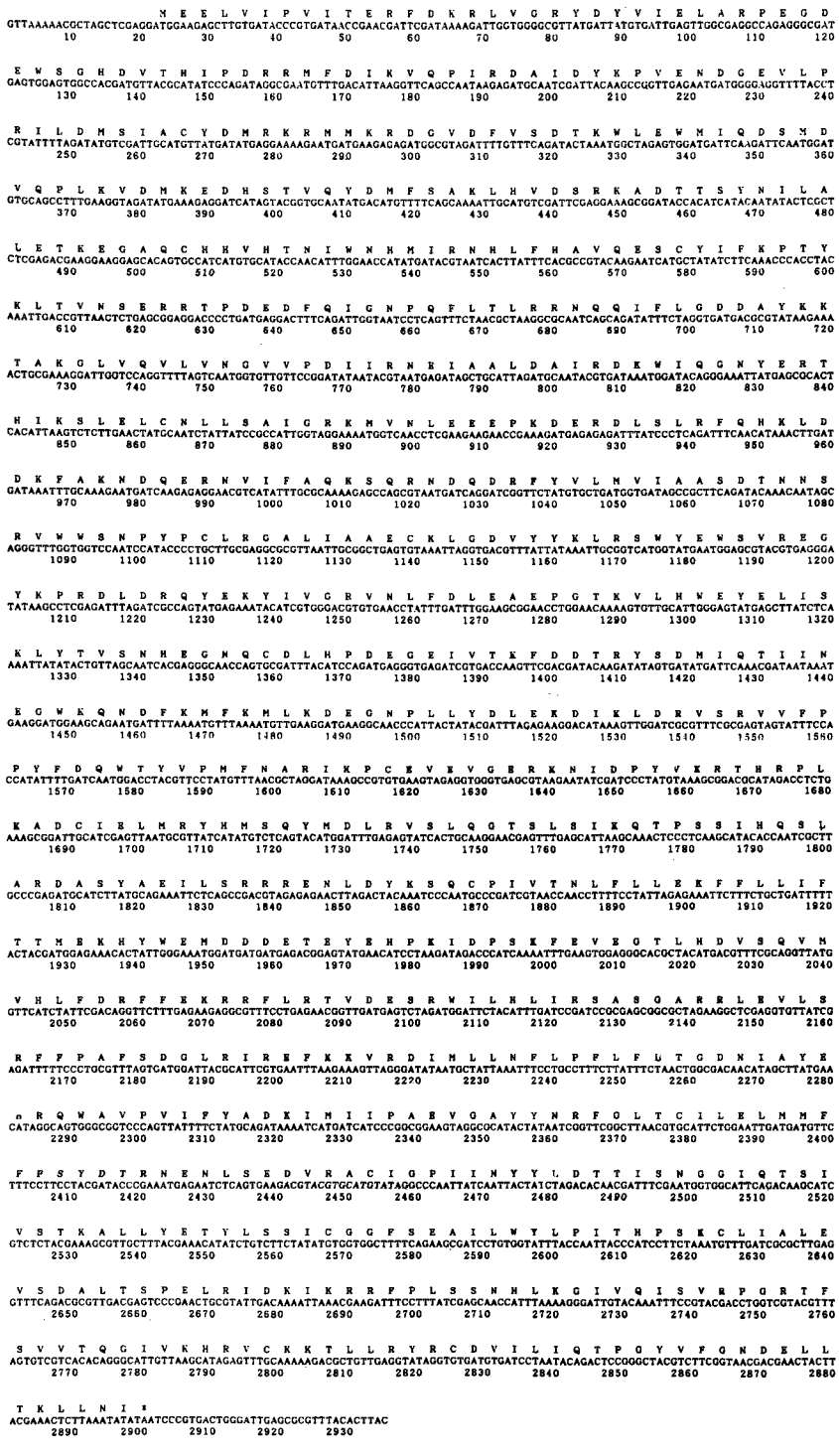

Fig. 2. The nucleotide sequence of the cloned L3 gene of BTV-17. The open reading frame begins at positions 22 to 24 and is terminated by the TAG codon at positions 2888 to 2900. The single-letter amino acid codes are shown above their respective DNA codons.

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BTV-10  MEEFVIPVFS ERDIPYSLIN HYPLAIQIDV KVDDEGGKHN LIKIPESDMI DVPRLSIIEA LYNRPKRNDG   69
BTV-11  ********** *TE******S *****VRTM* *OAMVDEG*D VV******** ****V**V** *AAL*T****
BTV-17  *********Y* *DE***A**S R******TN* *IEDVE**** VV******** *O*L*T*V** M**L*A****
BTV-13  ***L****IT **-FDKR*VG R*DYV*ELAR PEG**WSG*D VTH**DRR*F *IKVQP*RD* ID*K*VE***

BTV-10  VVVPRLLDIT LRAYDNRKSA KNAKGVEFMT DTKWMKWAID DKMDIQPLKV TL-DNHCSVN HQLFNCIVKA  139
BTV-11  I********* *****D**AM *S*R****** NA******** *R******** AI-*D*NA** **********
BTV-17  I********* *****D***T *S*R*Q**** NAR******* *R******** **-*HY**** ******V***
BTV-13  E*L**I**MS IAC**M**RM MKRD**D*VS ****LE*M*Q *S**V***** DMKED*ST*Q YDM*SAKLHV

BTV-10  RSANADTIYY DYYPLE-NGA KRCNHINLDL LRSLTTTEMF HILQGAAYAL KTTYELVAHS ERENMSESYQ  209
BTV-11  *P*****V** S*F**R-DKV *K******** **G******* *M******C* *SS***ITN* **N*TF*T*A
BTV-17  NA******** **F***-DYK ********** *****NM*L* *A******SI *SS******Y* **GSLE*T*V
BTV-13  D*RK***TS* NILA**TKEG AQ*H*VHTNI WNHMIRNHL* *AV*ESC*IF *P**K*TVN* **RTPD*DF*

BTV-10  VGTQRWIQLR KGTKIGYRGQ PYERFISSLV QVIIKGKIPD EIRTEIAELN RIKDEWKNAA YDRTEIRALE  279
BTV-11  P*VHNR*R*V R**R***K*F A*S**V**** **R*Q*OT*P **VDD**R** E*RT**T**Q F*S*K*****
BTV-17  **QPK**H*T R**R**NS*L S*******M* **SVN***** **AN***Q** **RA**IT*T ***GR*****
BTV-13  I*NPQFLT** RNQQ*FLGDD A*KKTAKG** **LVN*VV** I**N***A*D A*R*K*IQGN *E**H*KS**

BTV-10  LCKILSAIGR KMLDVQEEPK DEMALSTRFQ FKLDEKFIRT DQEHNVIFKV GGSATDDGRF YALIAIAGTD  349
BTV-11  ********** ***NTH**** ***D****** ****D**KK* *S**I***N* *AP**HE*** *******A**
BTV-17  **S***T*** ***NTH**** ***D****** *******N*A *S******G* R*P***E*** *******A**
BTV-13  **NL****** **VNLE**** **RD**L*** H***D**AKN ***RNV**AQ KSQRN*QD*S *V*MV**AS*

BTV-10  TQQGRVWRTN PYPCLRGALI AAECELGDVY FTLRQTYKWS LRPEYGQRER PLEDNKYVFA RLNLFDTNLA  419
BTV-11  **R******* ********** ****Q***** HQ***V**** **QD**RT*V ***N*****S *I****S**E
BTV-17  **K******* *********V ********** S***RVHT** *******H*R Q**N*****N *I****S***
BTV-13  *NNS***WS* ********** ****K***** YK**SW*E** V*EG*KP*DL DRQYE**IVG *V****LEAE

BTV-10  VGDEIIHWRY EVYQPK-ETT HDDGYICVSQ KGDDELLCEV DEDRYKEMFD RMIQGGWDQE RFKLHNILT-  489
BTV-11  ***QVV**K* *IDG*A-*** Y*N****KTE RE*G**V*KI S*EK**T*L* ********** ****YSV**-
BTV-17  ***Q****** **KASA-*** Y*S**M*RHE AEE*****KI N**K****L* ********** *********-
BTV-13  P*TKVL**E* *LISKLYTVS NHE*NQ*DLH PDEG*IVTKF *DT**SD*IQ TI*NE**K*N D**MFKM*KD

BTV-10  EPNLLTIDFE KDAYLGARSE LVFPPYYDKW INSPMFNARL KIARGEIATW KADDPWSNRA VHGYIKTSAE  559
BTV-11  D********* *****NE*** F*L*S*F*O* *Y******** R*TH**NK*V *F**V*A*T* *P**V*A*T*
BTV-17  D********* *****NS*** **L*D*F*** *S******** R*TK***G*S *K****N*** *R****PL**
BTV-13  *G*P*LY*L* **IK*DRV*R V*****F*Q* TYV******I *PCEV*VGER *NI**YVK*I HRPLKADCI*

BTV-10  SLEYALGPYY DLRLQLFGDT LSLGQRQSAV FEHMAQQDDF STLTDYTKG- ---RTVCPHS GGTFYTFRKV  628
BTV-11  *P*****Q*F *T*I**Y**A ***K*S**** *Q*QS**E** PV**S*A**- ---DV***** **AL******
BTV-17  **DFV***** ****LF*DE* ***K*E**** *QYLS*L*** PA**-QLR*- ---DA***** **AL******
BTV-13  LMR*HMSE*M ***VS*Q*TS **IK*TP*SI HQSL*RDASY AEILSRRREN LDYKSQ**-I VTNLFLLE*F

BTV-10  ALIILSNYER LDPSLHEGRE HETYMHPAVN DV-FRRHVLE MKDFSQLICF VFDYIFEKHV QLRNAKEARR  695
BTV-11  **MLMA**** *S*D****M* DH**T**SIG GANQ-KRI** *R******** II*****R*D ***DMR****
BTV-17  **FLIG***K *S*D****M* *QR*V**ST* *TYQ-KRV** ***SC**T** *I******RE ***DT****Y
BTV-13  F*L*FTTM*K HYWEMDD--D ETE*E**KID PSK*EVEG-T LH*V**VMVH L**RF***RR F**TVD*S*W

BTV-10  IIYLIQNTSG AYRLQVLRAE FPNFLKHVMN LRDVKRICDL NVINFFPLLF LVQDNISYWH RQWSIPMILF  764
BTV-11  *L**V*SLGE PQ*****SVA S***SRYFLK *K**Q**S** *****L**** *I******** ***AV*****Y
BTV-17  *V****SLT* TQ**S***ST ****FQRLLM *KEI*FVR** *****L**M* **H******* *******V**
BTV-13  LL***RSA** *R**E**SRF **A*-SDGLR **EF*KVR*L MLL****F** *TG***A*E* ***AV*V*FY

BTV-10  DQVIRLIPVE VGAYANRFGL KSFFNFIRFH PG-DSKKRQD ADDTHKEFGS ICFEYYTTTK ISQGEIDVPV  833
BTV-11  *DT*K***** *********I ******T*** **-*A****K *********L *S*N**AN** *A**GVHT**
BTV-17  *DT*K***** *********F ***M**T*** **-EL**K*I *E*I*****V VA*****N** ****NVHT**
BTV-13  ADK*MI**A* ****Y***** TCILELMM*F *SY*TRNENL SE*VRACI*P *-IN**LD*T **N*G*QTSI

BTV-10  VTSKLDTLKL HVASLCAGLA DSLVYTLPVA HPKKSIVLII VGDDKLEPQI RSEQIVNKYY YSRRHISGVV  903
BTV-11  **T******I *LS******* **V******* ****C***** ********HV ****V*S*** F**K*V****
BTV-17  M*T*M*V*RV *LS******* **V******* ****C***** ********HT ******SR*N ***K**C*I*
BTV-13  *ST*ALLYET YLS*I*G*FS EAILWY**IT **S*CLIALE *S*ALTS*EL *IDK*KRRFP L*SN*LK*I*

BTV-10  SICVNQGGQL KVHSMGITRH RICDKSILKY KCKVVLVRMP GHVFGNDELM TKLLNV                959
BTV-11  ***IG*ND** **Y*S**V** ***E*F**R* *******K** *Y********* ******
BTV-17  *VTIG*NS** R**TS**VK* *V***F***H ****I***** *Y********* ******
BTV-13  Q*S*RP*RTF S*VTQ**VR* *V*K*TL*R* R*D*I*I*N* *********L *****I
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Fig. 3. Amino acid sequence comparisons of VP2 proteins of four BTV serotypes using the BTV-10 sequence as a reference (Purdy *et al.*, 1985). The deduced amino acid sequences of the BTV-11 L2 gene product, the BTV-17 L2 gene product and the BTV-13 L2 gene product were compared with the previously published L2 gene product of BTV-10 (Purdy *et al.*, 1985). Alignment of the 959 amino acid sequences was performed by incorporating a number of gaps. The conserved amino acids are indicated by stars and variable regions by lines below the amino acids.

whereas at least 13 gaps were required to align their sequences with the VP2 of BTV-13. Numerous amino acid differences were evident in the BTV-13 sequence by comparison with the corresponding proteins of the other three virus serotypes. While 70% of the amino acid sequences of the other three BTV serotypes are conserved, only 39 to 40% of the aligned amino acids were identical for BTV-13 and BTV-10, BTV-13 and BTV-11, or BTV-13 and BTV-17. Many of the differences represented single nucleotide and amino acid changes; however, there were several regions of consecutive nucleotide and amino acid dissimilarity (Fig. 3). As a result, a number of regions exhibited clustered amino acid changes, for example amino acid residues 14 to 66, 155 to 178, 430 to 451, 585 to 608 and 632 to 663. Several of these variable regions involved mainly non-conservative amino acid changes (e.g. residues 155 to 178 and 585 to 608). For all four viruses the most homologous portion of the VP2 molecules occurred near their carboxy termini (residues 943 to 959), where 15 to 26 residues were identical or conserved among the four sequences. It is possible that this portion of the VP2 molecule has a functional requirement, limiting its evolution. Another conserved region was in the middle section of the molecule (residues 354 to 379). A feature of the VP2 protein comparisons of the four viruses was the positions of their cysteine, proline and glycine residues. Whereas the positions of all three amino acids of the VP2 polypeptides of BTV-10, -11 and -17 were highly conserved (75 to 85%), for the BTV-13 VP2, only 56% of the cysteines, 62% of the prolines and 59% of the glycines were in positions identical to those of BTV-10 VP2.

The elucidation of the molecular basis of serotype diversity is an important goal in bluetongue virus research. In this report we have presented the complete L2 gene sequence of BTV-13 and compared the predicted amino acid sequence with those of three other BTV serotypes in the belief that information about the regions of variations and their hydrophilic nature would indicate the type-specific epitopes and/or group-specific epitopes.

Our earlier findings by oligonucleotide fingerprint analysis as well as by Northern blot hybridization studies have indicated that the L2 gene of U.S.A. BTV-13 differs considerably in comparison to the L2 genes of the other three U.S.A. serotypes. From an evolutionary viewpoint this may indicate that BTV-13 evolved earlier from a common ancestor. Several aspects of the BTV-13 L2 RNA sequence are worthy of comment. First, unlike the other BTV L2 genes (Purdy *et al.*, 1984; Ghiasi *et al.*, 1987) only seven nucleotides at the 5′ non-coding region are conserved. Therefore, it is unlikely that the 5′ non-coding region has additional functions other than recognition by the viral transcriptase/replicase. The 3′ non-coding region is more extensively conserved than the 5′ non-coding region, a situation similar to that noted for other genes of the different BTV types (Purdy *et al.*, 1984, 1985, 1986; Ghiasi *et al.*, 1985; Lee & Roy, 1986). The reasons for the length of this sequence conservation are not known, possibly this region of the RNA is involved in viral morphogenetic events.

Both the composition and sequence comparisons of the predicted amino acids of four L2 genes have indicated that BTV-13 is very different from the other three U.S.A. viruses. Nevertheless, the common phylogenetic origin of all four viruses was revealed by the diagonal lines when the predicted amino acid sequence of BTV-13 L2 gene product was compared with that of each of the L2 polypeptides of the other three viruses (Fig. 4). The distribution of the homologous sequences was not evenly spread through the molecules, as is apparent from these analyses. As shown by the arrows, there was at least one large gap for each pair. For the BTV-17 and BTV-13 pair, there was an additional large gap in the diagonal line which indicated that BTV-13 was more distantly related to BTV-17 than to BTV-10 and BTV-11.

Since the BTV VP2 protein constitutes the major target of the host immune response, the function of the surface of the protein is to present a unique structure which might give an evolutionary advantage to the protein while preserving the three-dimensional structure. If this is so, only the general properties of a protein would have to be maintained (i.e. shape, interactions with other proteins, possibly charge balance or hydrophilicity versus hydrophobicity). We noticed that although similarities in amino acid composition of VP2 for BTV-13 with those of the other three viruses were comparatively weak overall, the hydropathic profiles and the distribution of charged residues of all the proteins were quite similar with a few exceptions (as shown in Fig. 5). For example, all four VP2 molecules shared at least 12 regions of similar or only

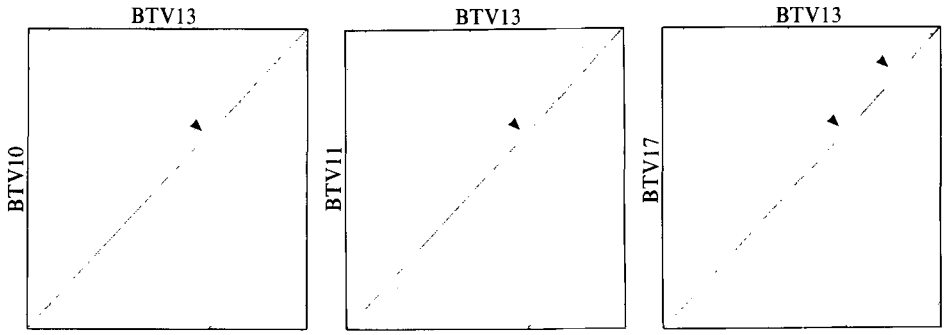

Fig. 4. Diagon analyses. The complete amino acid sequences of predicted L2 gene product of BTV-13 was compared pairwise with those of BTV-10, BTV-11 and BTV-17 using the Diagon program of Staden (1982). Homologies are indicated by diagonal lines. For the protein comparison an 11 amino acid span and a proportional index of 131 was employed.

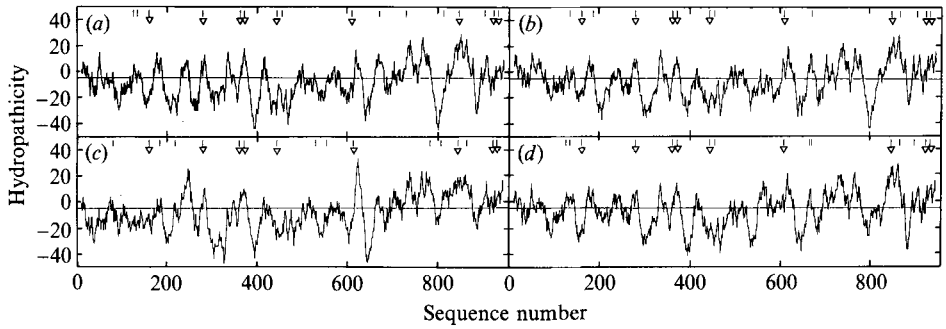

Fig. 5. Hydropathic plot and distribution of cysteine for the predicted L2 gene products of (*a*) BTV-10, (*b*) BTV-11, (*c*) BTV-13 and (*d*) BTV-17. The regions of the predicted proteins with a net hydrophobicity (areas above the centre line) or hydrophilicity (areas below the centre line) as well as the distribution of cysteine residue (vertical bars) are displayed (Kyte & Doolittle, 1982). The plot involves a span setting of 21 amino acids. Positions of conserved cysteines are indicated by arrows.

slightly dissimilar hydrophilic profiles which include amino acid residues 14 to 60, 100 to 170, 192 to 215, 250 to 274, 291 to 359, 385 to 409, 430 to 491, 509 to 559, 641 to 664, 680 to 700, 796 to 810 and 880 to 900. Several hydrophobic regions (e.g. amino acid residues 610 to 640, 725 to 795, 840 to 870) were also conserved among the four protein molecules. In addition, as shown in Fig. 5, there were at least nine cysteine positions that were conserved among the four VP2 molecules. Perhaps only eight to ten disulphide linkages were present in the VP2 molecule.

As yet no clear sites of genetic variation have emerged from these comparisons. An attempt to locate the antigenic sites of the protein by analysing computer-produced structural 'cartoons' failed. It is obvious that more detailed knowledge concerning protein structure is needed. To this end future experiments involving site-specific mutagenesis of the VP2 proteins, as well as synthetic peptide or subunit peptides expressed in appropriate vectors are planned in order to obtain insight into the role of these antigens.

We thank the staff of the NERC Institute of Virology especially C.D. Hatton for photography, Mrs J. Oswell for typing and Dr V.C. Emery for computer analysis. This work was supported by the U.S. Department of Agriculture grant no. 86-CRCR-1-2189, ARS/APHIS grant 58-2349-6-011 and EEC contract BAP.0120.UK.

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(*Received 8 May 1987*)