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Molecular Basis of Bluetongue Virus Neutralization

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Molecular and serological analyses of bluetongue virus serotypes 10 and 11 and their intertype reassortants indicate that the viral RNA segment L2 codes for the serotype-specific antigen. Individual RNA segments of parental and reassortant viruses were characterized by oligonucleotide fingerprint analyses. Analyses of their virion polypeptides by Cleveland peptide mapping (Cleveland et al., J. Biol. Chem. **252**:1102–1106, 1977) demonstrated that the L2 gene segregated colinearly with the viral P2 protein, implicating it as the antigen that is responsible for the viral serotype specificity.

Bluetongue virus (BTV) is the prototype virus of the genus *Orbivirus* in the family Reoviridae. The genome of BTV consists of 10 segments of double-stranded RNA (dsRNA) (15). These 10 RNA segments have been categorized as large (L1 through L3), medium (M1 through M3), or small (S1 through S4) on the basis of their molecular weights (12). In the virus particle the dsRNA is surrounded by a double-capsid shell, the outer capsid consisting of two polypeptides (VP2, VP5) and the inner core consisting of two major (VP3, VP7) and three minor (VP1, VP4, VP6) polypeptides (2, 9, 14). It has recently been demonstrated by immune precipitation techniques that VP2 is the serotype-specific antigen (6). Which viral RNA segment codes for VP2 is not known.

Mustoe and associates (10) have used genetic crosses between temperature-sensitive mutants of reoviruses to obtain reassortant viruses to identify the information content of the individual reovirus genes. Using wild-type virus crosses involving distinguishable BTV serotypes, we have determined that the L2 gene of BTV cosegregates with the viral antigens responsible for type specificity of the virus. The results obtained also demonstrate that the L2 RNA segment codes for polypeptide VP2.

MATERIALS AND METHODS

Materials. Acrylamide and bisacrylamide were obtained from Serva Laboratories through Accurate Chemical Corp., Hicksville, N.Y. Sankyo RNase T_1 was obtained from Calbiochem, La Jolla, Calif. Sodium $^{32}P_i$, [^{35}S]methionine, and [^{3}H]leucine were purchased from ICN Pharmaceuticals Inc., Irvine, Calif. *Staphylococcus aureus* V8 protease was purchased from Miles Laboratories, Inc., Elkhart, Ind.

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Viruses and cells. BTV serotypes 10 (BTV-10) and 11 (BTV-11) came from the collection of the Arthropod-Borne Animal Diseases Research Laboratory, Denver, Colo., courtesy of T. L. Barber and T. E. Walton. Each virus was plaque cloned in our laboratory, using monolayers of BHK-21 cells.

Mixed virus infections and isolation of reassortant viruses. Procedures used to produce and recover reassortant viruses by dual wild-type virus infections have been described previously (3, 16). In short, BHK-21 monolayer cultures (5×10^6 cells) were coinfected with the two wild-type viruses, each at 5 PFU per cell. After 48 h of incubation in Eagle minimal essential medium at 37°C, progeny viruses were harvested and then plated on BHK-21 cell monolayers. Well-separated plaques were picked and then individually passaged in BHK-21 cells. For genotype analyses, each cloned progeny virus was grown in the presence of ^{32}P (200 μCi/ml).

Preparation and purification of dsRNA. The recovery of ^{32}P-labeled dsRNA from infected cells has been described previously (12, 13).

RNA gel electrophoresis and isolation of individual dsRNA species. Purified dsRNA preparations were electrophoresed in slab gels of 8% polyacrylamide (12). For oligonucleotide fingerprinting of individual segments, RNA was resolved by electrophoresis in 1% (wt/vol) agarose gels (12, 13). The location of ^{32}P-labeled RNA segments in the gels was determined by autoradiography, and each segment was recovered and purified as described previously (12, 13).

Oligonucleotide fingerprinting of dsRNA species. Methods for the denaturation and digestion of dsRNA with RNase T_1 and the two-dimensional electrophoretic analyses of the derived oligonucleotides have been described previously (12, 13).

Analysis of radiolabeled infected cell proteins. Cultures of 5×10^6 cells were infected with 1 to 10 PFU of virus per cell. After 12 h of incubation, the infected monolayers were washed with Eagle minimal essential medium lacking leucine, or methionine. The medium was then replaced with the same medium containing 25 μCi of [^{35}S]methionine. After 3 to 4 h of incubation at 37°C, the medium was removed and the cells were washed and then lysed by the addition of 1 ml of 0.06

M Tris-hydrochloride, pH 6.8, containing 2% (wt/vol) sodium dodecyl sulfate, 2% (vol/vol) 2-mercaptoethanol, 0.3 M urea, and 10% glycerol (8). For analytical and preparative purposes, the labeled infected cell proteins were routinely electrophoresed in 9% polyacrylamide gels, using the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis system of Laemmli (8). The gels were fixed, and the viral specific polypeptides were visualized by autoradiography.

Peptide analysis of ^{35}S-labeled viral proteins. Gel slices containing [^{35}S]methionine-labeled viral polypeptides were eluted in 0.125 M Tris-hydrochloride, pH 6.8, containing 0.1% sodium dodecyl sulfate and 1 mM EDTA. Peptide mapping by limited proteolysis with *S. aureus* V8 protease was carried out as described previously by Cleveland and associates (1). The resulting peptides were analyzed by electrophoresis in 15% polyacrylamide slab gels (8). The gels were dried in vacuo, and the peptide bands were visualized by autoradiography.

Serological characterization of BTVs. Procedures for virus serotype identification involved infecting a monolayer culture of BHK-21 cells with 0.1 ml of a virus suspension containing 10^3 to 10^4 PFU, using an overlay of 0.5% agarose, Eagle minimal essential medium and 5% fetal calf serum. After the overlay had solidified, four 0.25-inch (0.635-cm) paper disks, each previously soaked in BTV-10-, BTV-11-, bluetongue viruse serotype 13 (BTV-13), or bluetongue virus serotype 17 (BTV-17)-immune sera, were placed on the agarose surface (11). After 24 to 48 h of incubation, the disks were removed and the plates were stained with neutral red and observed for viable cells. Immune sera were kindly provided by M. M. Jochim (Arthropod-Borne Animal Diseases Research Laboratory). The presence of a red halo of viable cells around the position of an individual paper disk was taken as evidence of antibody protection of cells infected with the homologous virus serotype.

RESULTS

Isolation of recombinant viruses and analyses of their RNA species by gel electrophoresis. For the in vitro BTV recombination analyses the Idaho isolate of BTV-11 (BTV-73-27S) and the U.S.

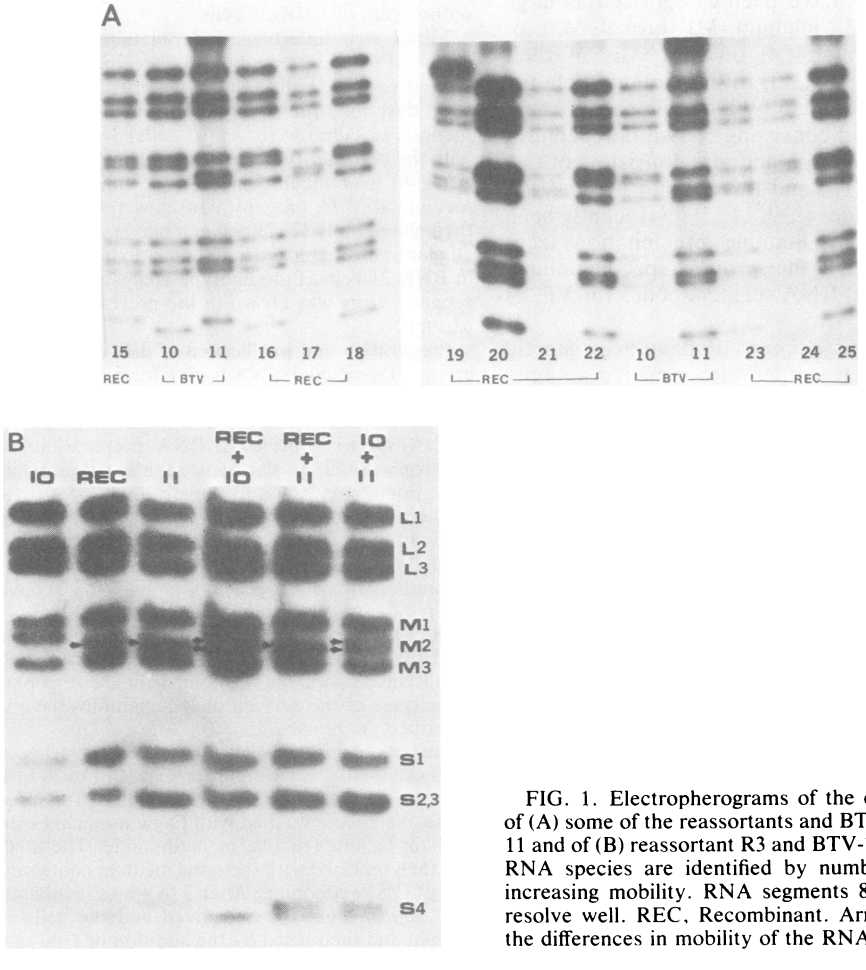

FIG. 1. Electropherograms of the dsRNA species of (A) some of the reassortants and BTV-10 and BTV-11 and of (B) reassortant R3 and BTV-10 and BTV-11. RNA species are identified by number in order of increasing mobility. RNA segments 8 and 9 did not resolve well. REC, Recombinant. Arrowheads show the differences in mobility of the RNA segments.

TABLE 1. Serotype analyses of 48 progeny viruses obtained from wild-type BTV-10 and BTV-11 crosses

Virus[a]	Presence (+) or absence (−) of antibody protection in:					
	Genotype				Serotype	
	BTV-10		BTV-11		BTV-10	BTV-11
	M2	S4	M2	S4		
Parent BTV-10	+	+	−	−	+	−
Parent BTV-11	−	−	+	+	−	+
Group A	+	+	−	−	+	−
Group B	−	−	+	+	+	−
Group C	−	−	+	+	−	+
Group D	+	+	−	−	−	+
Group E	−	+	+	−	+	−
Group F	−	+	+	−	−	+
Group G	+	−	−	+	+	−
Group H	+	−	−	+	−	+

[a] All of the following progeny marked with an asterisk (*) were further analyzed for RNA and proteins. Group A included 8 cloned progeny, R18, R24, R41, R47, R50, R51, R58, and R59; no progeny were found representing group B; group C included 21 cloned progeny, R1, R2, R7 through 10, R12, R14, R17, R19, R21, R23, R29, R33, R42, R46, R48, R49, R52, R55, and R57; group D included 1 cloned progeny, R16*; group E included 1 cloned progeny, R3*; group F included 8 cloned progeny, R4*, R11, R13, R28, R43, R44, R45, and R56; group G included 6 cloned progeny, R6, R15, R20, R22, R40, and R53; and group H included 3 cloned progeny, R5, R25, and R60.

prototype strain of BTV-10 (BTV CA8; Calif.) were used (12). These viruses were chosen since the electrophoretic mobilities of their M2 and S4 segments are distinguishable (Fig. 1A). None of the other BTV-10, BTV-11, BTV-13, or BTV-17 isolates that we have so far analyzed has more differences in RNA segment mobilities than these two isolates (unpublished data). BHK-21 monolayer cell cultures were coinfected with equivalent multiplicities of the two wild-type viruses. Progeny virus plaques were picked and individually passaged in BHK-21 cells to obtain virus stocks. A total of 48 cloned progeny virus stocks were then grown for 48 h in the presence of ^{32}P (200 μCi/ml), and the viral dsRNA was extracted from infected cells as described previously (12, 13). Purified extracts of dsRNA were resolved by 7.5% Laemmli polyacrylamide gel electrophoresis, and their RNA mobilities were compared with those of the two parental viruses (12). Some reassortant viruses were identified by this technique due to the segregation of the M2 and S4 RNA species (see Table 1 and R25 on Fig. 1A). When dsRNA preparations from one of these reassortants (R3) were coelectrophoresed with RNA of BTV-10 or BTV-11 in a polyacrylamide slab gel, it was evident that both the reassortant and BTV-11 shared segment M2. It was also evident that the reassortant had an S4 segment of BTV-10 (Fig. 1B).

Serotype analyses of BTV reassortants. The 48 progeny virus stocks were screened serologically (Table 1), using a modified plaque inhibition method test (11). The results obtained were correlated to the M2 and S4 genotype analyses; these results allowed categorization of progeny into groups (Table 1). Progeny represented by groups A and C resemble the parental viruses both in terms of their M2 and S4 genotypes and their serotype specificities. However, both groups may contain reassortant viruses with substitutions in other RNA segments. All other groups (19 viruses) represent reassortant viruses on the basis of the segregation of M2 and S4 RNA species and serotype specificities. It was concluded from the fact that the serotype specificity independently of the M2 or S4 segments (Table 1) that neither of their gene products is involved in virus neutralization. No reassortant was obtained with M2 or S4 segments of BTV-11 and serotype specificity of BTV-10 (Table 1).

TABLE 2. Comparison of the large oligonucleotides of the 10 RNA segments of reassortant R16 with those of BTV-10 and BTV-11

RNA segment	No. of RNA segment large oligonucleotides that were:			Parental origin of R16
	Compared	Similar to those of BTV-10	Similar to those of BTV-11	
1	25	24	14	BTV-10
2	30	Dissimilar	25	BTV-11
3[a]	30	27	25	BTV-10
4	28	28	17	BTV-10
5	15	14	Dissimilar	BTV-10
6	35	33	15	BTV-10
7	23	20	9	BTV-10
8	25	24	5	BTV-10
9	20	19	9	BTV-10
10	23	21	Dissimilar	BTV-10

[a] BTV-10 (prototype strain) and BTV-11 (Idaho strain) are almost identical on RNA segment 3 (13).

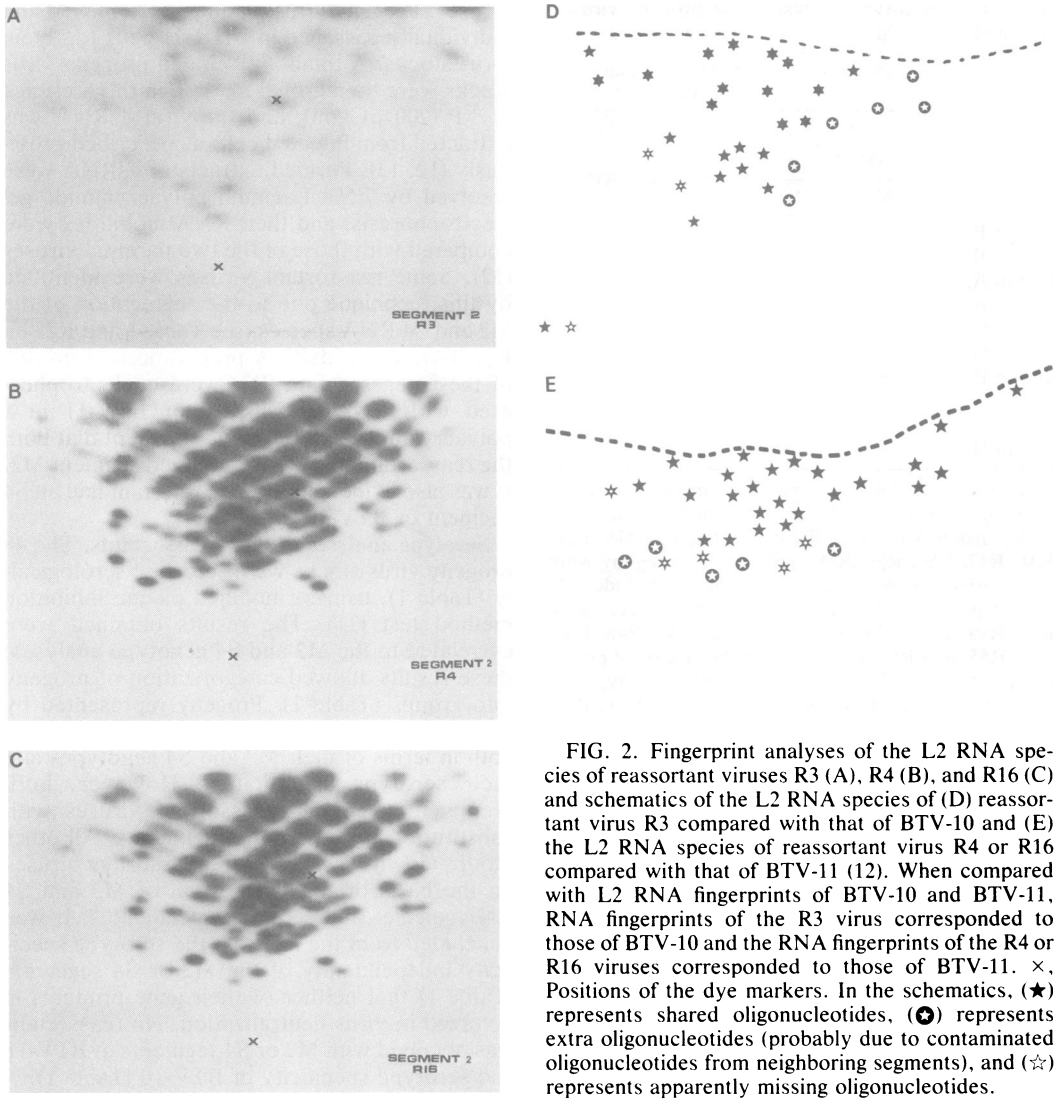

FIG. 2. Fingerprint analyses of the L2 RNA species of reassortant viruses R3 (A), R4 (B), and R16 (C) and schematics of the L2 RNA species of (D) reassortant virus R3 compared with that of BTV-10 and (E) the L2 RNA species of reassortant virus R4 or R16 compared with that of BTV-11 (12). When compared with L2 RNA fingerprints of BTV-10 and BTV-11, RNA fingerprints of the R3 virus corresponded to those of BTV-10 and the RNA fingerprints of the R4 or R16 viruses corresponded to those of BTV-11. ×, Positions of the dye markers. In the schematics, (★) represents shared oligonucleotides, (✪) represents extra oligonucleotides (probably due to contaminated oligonucleotides from neighboring segments), and (☆) represents apparently missing oligonucleotides.

Oligonucleotide fingerprint analyses of the individual RNA species obtained from three reassortants. Although the RNA gel electrophoresis and serotype screening procedures indicated that reassortants were produced, they did not show which RNA coded for the antigens recognized by neutralizing antisera. Since for all progeny the origins of eight of their RNA species were unknown, three reassortants, R3, R4, and R16 (representing groups E, F, and D, respectively, in Table 1), were selected for more detailed genotype analyses by individual RNA segment oligonucleotide fingerprinting (12). When individual RNA fingerprints of these three reassortant viruses were compared with corresponding RNA fingerprints of the parental viruses, the following deductions were made. (i) For reassortant R3 (group E, a BTV-10) all RNA segments came from BTV-10, except the M2 segment. (ii) For reassortant R16 (group D, a BTV-11) all RNA segments were derived from BTV-10, except segment 2 (L2) as shown in Table 2; this result indicated that the L2 RNA codes for the gene products recognized by neutralizing antibodies. (iii) For reassortant R4 (group F, a BTV-11) only the S4 segment was derived from BTV-10; all others came from BTV-11.

Figure 2A, B, and C show the fingerprint analyses of the L2 segments of the three reassortant viruses analyzed. By comparison with previously published data (12) it was evident that the L2 RNA of the R3 reassortant had a fingerprint like that of BTV-10 (Fig. 2D). Fingerprints of the L2 RNA segments of the other two

viruses were similar to each other and to that of the L2 RNA of BTV-11 (Fig. 2E; 12).

Analyses of the polypeptides induced in cells infected with selected reassortant progeny BTV viruses. Since comparisons of the proteins of parental and reassortant viruses can be used to determine coding assignments of the viral RNA species, analyses of the proteins induced by the two parental and three reassortant viruses, R3, R4, and R16, were undertaken. Cultures of 5×10^6 BHK-21 cells were infected with each virus or mock infected, and the [^{35}S]methionine-labeled proteins were recovered and analyzed by 9% polyacrylamide gel electrophoresis. By comparison with the infected cells, 10 virus-induced polypeptides were detected in the cell extracts (Fig. 3). Although the polypeptides are numbered according to their sizes (5), it is not known which is the product of which viral RNA segment (4). Except for polypeptide VP5 of BTV-10 and BTV-11 viruses, no size differences were detected between the proteins induced by the two parental viruses. Polypeptide VP5 of the R16 reassortant exhibited a similar electrophoretic mobility to that of BTV-10, unlike that of BTV-11 or the other two reassortants, R3 and R4.

Since the only BTV-10 RNA segment present in the R16 reassortant was the M2 segment, the data indicate that M2 RNA codes for protein VP5. Reassortant R3, which had a polypeptide VP5 derived from BTV-11, serotyped as BTV-10. These results agree with the conclusion that polypeptide VP5 does not contain the epitopes recognized by neutralizing antibodies (6).

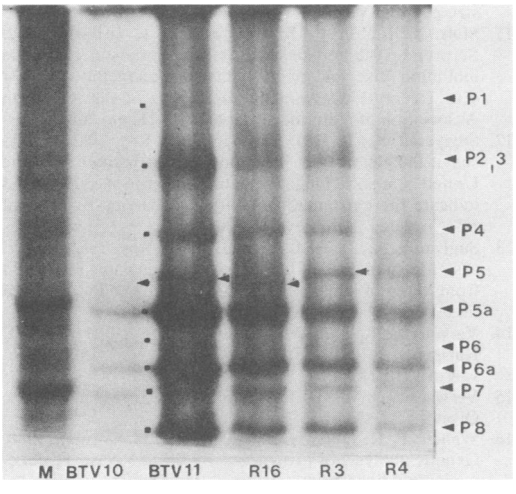

FIG. 3. Autoradiogram of a 9% polyacrylamide slab gel of [^{35}S]methionine-labeled polypeptides recovered from mock-infected cells (M) or cells infected with BTV-10, BTV-11, or reassortants R3, R4, or R16. Polypeptides (P) are numbered in accordance with data previously published by Huismans (5). Arrowheads highlight the differences in mobility of polypeptides. Each polypeptide is dotted to show banding.

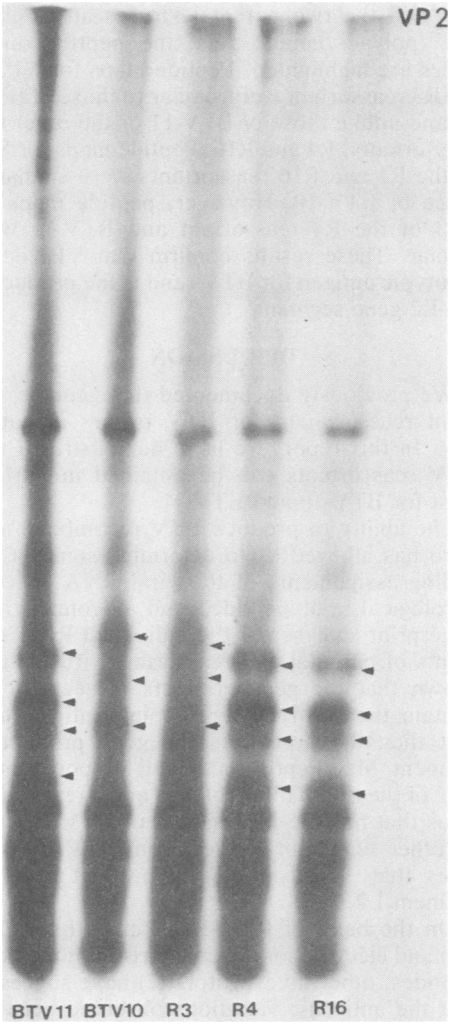

FIG. 4. Limited proteolysis of [^{35}S]methionine-labeled polypeptides P2 of BTV-11, BTV-10, and reassortants R3, R4, and R16 digested with *S. aureus* V8 protease. Arrows indicate the peptides similar to the parental serotypes.

Limited proteolytic analyses of BTV-induced polypeptides. Peptide analyses of the virus-induced polypeptides were undertaken by limited proteolysis with *S. aureus* V8 protease by the procedures of Cleveland and associates (1). Since VP2 and VP3 have almost identical mobilities, they were eluted from the gel and run on a second gel for twice the time to obtain a better separation. Figure 4 shows autoradiograms of partial digests of polypeptides 2 of R3, R4, and

R16 and the two parental viruses analyzed in 15% polyacrylamide gels; the peptide differences are highlighted. Peptide maps for VP2 of the R3 reassortant were similar to those of BTV-10 and unlike those of BTV-11 or the other two reassortants, R4 and R16. Peptide maps for VP3 of the R3 and R16 reassortants were similar to those of BTV-10. However, peptide maps for VP3 of the R4 reassortant and BTV-11 were similar. These results confirm that VP2 is the serotypic antigen for BTVs and is the product of the L2 gene segment.

DISCUSSION

We previously documented that genome segment reassortment for BTV occurs in nature (12). In this report we have demonstrated that BTV reassortants can be obtained in vitro at least for BTV-10 and BTV-11.

The ability to produce BTV recombinants in vitro has allowed us to determine some of the coding assignments of the viral RNA species. Serological, polypeptide, and oligonucleotide fingerprint analyses of the individual RNA segments of parental and reassortant viruses have shown that the gene products of segment L2 contain the serotype-determining antigens and that the VP5 protein is the gene product of segment M2. Peptide mapping of polypeptide VP2 of the two BTV serotypes and a reassortant virus that has the L2 segment of BTV-11 (with all other segments derived from BTV-10) indicates that VP2 is the gene product of RNA segment L2.

On the basis of RNA-RNA cross hybridization and electrophoretic comparison of the polypeptides, other investigators (7) have suggested that the antigenic variations observed between the different BTV serotypes are associated with the polypeptides of a viral outer capsid. Using immune precipitation studies, Huismans and Erasmus (6) recently suggested that polypeptide VP2 is probably the main determinant of the serotype specificity. Our data are in agreement with their conclusion.

The ability to produce BTV recombinants in vitro also allows questions about their virulence and pathogenicity in different host ranges to be addressed. Attempts to correlate individual, or groups of, viral RNA segments with virulence in ruminant hosts are currently in progress, using parental and reassortant BTV isolates.

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