Association of Bluetongue Virus Gene Segment 5 with Neuroinvasiveness

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Two strains (UC-2 and UC-8) of bluetongue virus were used to determine genetic factors influencing neuroinvasiveness. Reassortants were produced in vitro, and the parental origins of their genes were determined by polyacrylamide gel electrophoresis profiles and restriction endonuclease digestion. Gene segment 5 of UC-8 correlated with neuroinvasiveness of reassortants when inoculated subcutaneously into newborn mice.

Bluetongue virus (BTV), of the *Reoviridae* family, is a nonenveloped, insect-borne virus of ruminants. The BTV genome consists of 10 segments of double-stranded (ds) RNA, each of which encodes at least one polypeptide (16). The segmented genome permits genetic reassortment between two or more viruses infecting a single cell in vivo or in vitro (20). The outer capsid layer consists of two polypeptides, VP2 and VP5, encoded by gene segments 2 and 5, respectively (27). VP2 is responsible for serotype-specific neutralizing antibodies (12).

BTV causes severe brain malformations in fetal ruminants (2, 14) and experimentally infected newborn mice (18, 31). BTV-11 UC-8 causes encephalopathy when injected subcutaneously or intracranially into newborn mice, while BTV-11 UC-2 is encephalopathic only when injected intracranially (31). This difference relates to cell tropism and to the ability of the virus to invade the brain from the subcutaneous site (29). Inoculation of bovine fetuses at 240 days gestation with UC-8 resulted in the premature birth of weak calves with mild encephalitis, while UC-2-infected calves were normal (30). This study was performed to determine the genetic basis for the differences in neuroinvasiveness between BTV-11 UC-2 and UC-8.

Isolation and production of reassortants. BTV-11 UC-2 was isolated from a calf, and BTV-11 UC-8 was isolated from a deer (31). To produce reassortants, Vero cells were coinfectd with UC-2 and UC-8 at a multiplicity of infection of 2 for each virus. Progeny virus was harvested, and plaque was cloned (26). Viral dsRNA was prepared from infected Vero cells and visualized after electrophoresis on 13% discontinuous polyacrylamide gels (5) and silver nitrate staining (Fig. 1). Because of small differences in migration rates, it was possible to differentiate between genome segments of UC-2 and UC-8, except for segments 3 and 7. By allowing the gel to run for longer periods, segment 3 could also be differentiated (data not shown).

To differentiate the segment 7 genes, PCR was performed with two 20-mer oligonucleotide primers complementary to both ends of BTV-1SA gene segment 7 (28). Full-length

genome segment 7 PCR products were purified with a Gene Clean kit (Bio 101, Inc., La Jolla, Calif.) and were digested with either *Hae*III or *Xho*I (Boehringer Mannheim, Indianapolis, Ind.) for 1.5 h at 37°C. The restriction pattern was analyzed on a 1% agarose gel stained with ethidium bromide. *Xho*I cleaved segment 7 of UC-2 and reassortant 8 but did not cleave UC-8 or the other reassortants (Fig. 2). A similar pattern occurred after digestion with *Hae*III (data not shown).

More than 50 reassortant virus clones were analyzed. The UC-8 alleles were recovered preferentially over the UC-2 alleles, in agreement with a previous in vivo study (20). This suggests a selective advantage for the genes of the neuroinvasive UC-8 strain.

Neuroinvasiveness of reassortants. Six reassortants were chosen for assessment of neuroinvasiveness in newborn mice on the basis of their variable distributions of parental genes. UC-2, UC-8, or one of the six reassortant viruses was inoculated into one of eight groups of 18 to 25 newborn BALB/c mice. Twenty microliters of virus (containing 10⁴ PFU of virus diluted in sterile phosphate-buffered saline [PBS]) was inoculated subcutaneously into the dorsal neck within 24 h of birth. Mice were monitored daily postinoculation, and deaths occurring between 2 and 24 days were noted (Table 1). Sixteen mice were inoculated with 20 µl of sterile PBS as negative controls, none of which died within 24 days.

The percentages of inoculated mice dying after subcutaneous inoculation of these viruses are shown in Table 1. Reassortants possessing UC-8 genome segment 5 caused higher mortality than those with UC-2 segment 5. Only one of the reassortants with gene segment 5 derived from the neuroinvasive UC-8 strain showed an intermediate degree of neuroinvasiveness. This reassortant (reassortant 7) was the only neuroinvasive strain which had gene segments 8 and 9 from UC-2. Its attenuation may be due to modulation by these other genes, as seen in the Kemerovo group of orbiviruses (19). In that study, the atypical neurovirulence found in some reassortants was attributed to steric interactions between capsid proteins. Alternatively, the intermediate phenotype of reassortant 7 could also be due to a mutation in gene segment 5 or one or more other genes of this virus. Although gene 5 correlates with neuroinvasiveness in UC-2 and UC-8, other genes may be involved in other aspects of BTV virulence in other strains.

Brains were collected from all mice which died during the experiment, except for those that had been cannibalized. BTV

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FIG. 1. Polyacrylamide (13%) gel showing the electrophoretotype results for five of the reassortant studies. Lanes: 1, UC-2 run alone; 2, UC-2 run with UC-8; 3, UC-8 run alone; lanes 4 to 21, reassortants 6, 7, 8, 13, 14, and 15 run with UC-2 alone and with UC-8. The numbers on the left side correspond to the 10 genome segments of BTV.

was reisolated from these brains by cultivation on Vero cells. The migration pattern of the dsRNA extracted from each brain isolate was identical to that of the input virus when run on a polyacrylamide gel (data not shown).

Sequencing of segment 5 of UC-2 and UC-8. Full-length UC-2 segment 5 was amplified in a reverse transcriptase-PCR as described previously (1) with oligonucleotide primers designed from gene segment 5 of the prototype BTV-11 gene (6): upstream primer 5'-CCT GCA GTT AAA AAG TGT TCT CCT ACT-3' and downstream primer 5'-GCT GCA GTA AGT GTA AGG ATC TCC C-3'. PCR products were purified with the "Double Gene Clean" protocol as described by the manufacturer (Bio 101). Purified genes were ligated into the *Sma*I site of pUC-19 and transformed into *Escherichia coli* JM105. dsRNA from UC-8 was reverse transcribed and cloned into the *Sma*I site of pUC-13 as described previously (5).

pUC plasmids containing gene segment 5 of UC-2 or UC-8 were purified and sequenced with the Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Both

FIG. 2. Agarose (1%) gel (stained with ethidium bromide) showing restriction enzyme digestion of gene segment 7 of UC-2, UC-8, and the six reassortant viruses. Lanes: A to H, PCR-amplified segment 7 of UC-2, UC-8, and reassortant viruses 6, 7, 8, 13, 14, and 15, respectively, after being cut with *Xho*I. The molecular weight (MW) marker in the last lane is a 1-kb ladder from GIBCO BRL (Gaithersburg, Md.).

TABLE 1. Genome profiles and neurovirulence of reassortants

Virus strain	Parental origin of dsRNA segment ^a :										% of newborn mice killed ^b
	1	2	3	4	5	6	7	8	9	10	
UC-8	8	8	8	8	8	8	8	8	8	8	61
UC-2	2	2	2	2	2	2	2	2	2	2	5
6	2	8	8	2	8	8	8	8	8	8	63
8	8	8	8	8	8	8	2	8	8	2	56
14	8	2	2	8	8	8	8	8	8	2	52
7	8	8	8	2	8	8	8	2	2	2	24
13	8	8	8	2	2	8	8	8	8	2	5
15	2	8	2	2	2	2	8	8	8	8	0

^a Parental origin (UC-2 indicated by 2 and UC-8 indicated by 8) of each dsRNA genome segment of reassortant viruses as determined by polyacrylamide gel analysis or restriction enzyme digestion.

^b Percentage of newborn mice which died after subcutaneous inoculation of virus.

strands of each gene segment were sequenced by using at least two clones. The two genes were found to be 80% homologous, with changes distributed over the entire gene. The predicted amino acid sequences of VP5 of UC-2 and UC-8 were 97% identical, with 30 amino acid differences. These differences were in the N-terminal half of the peptide, with the largest group clustered in the first variable region of VP5 (32). Several of these amino acid differences cause major shifts in predicted secondary structure, which could potentially alter protein function.

No other biological properties have been definitively assigned to VP5 of BTV, although several have been proposed. No protection properties have been associated with purified or expressed VP5 (10, 15). However, addition of soluble VP5 increased the neutralizing immune response above that of VP2 alone (22), and VP5 may influence virus neutralization by influencing the conformation of epitopes of VP2 (4, 17). VP5 may play a role in binding to erythrocytes (21), affecting transport of virus to susceptible tissues. Studies with hybridization between the RNAs of virulent and avirulent strains of BTV indicated that genome segments encoding the outer capsid proteins (segments 2 and 5) are the ones that undergo changes during attenuation, indicating a role for VP5 in virulence (9).

VP5 is the only BTV protein which is glycosylated (33). Glycosylation of viral proteins has been associated with virus infectivity in Rous sarcoma virus (13), interactions with cellular receptors in human parainfluenza virus (8), and folding and transport in influenza virus (7), any of which may be important in BTV virulence. Neuroinvasiveness has been associated with the outer surface glycoproteins responsible for herpes simplex virus type 1, bunyavirus, and pseudorabies virus penetration into cells (3, 11, 25). In mammalian reoviruses, outer membrane protein μ 1 is important in viral neuroinvasiveness (24) and controls viral resistance to proteases and yield of virus from the cells of the central nervous system (23, 24).

Biological functions responsible for the differences in the neuroinvasiveness of UC-2 and UC-8 remain to be determined. Differences in tropism for neural cells do not appear to be involved (29). There may be a difference in the ability of the viruses to cross the blood-brain barrier, or UC-2 may be unable to replicate to a sufficiently high titer at a primary site before reaching the brain. Determination of the importance of outer capsid glycoprotein VP5 of BTV in neuroinvasiveness is a first step in understanding the molecular mechanisms involved in the pathogenicity of the virus.

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