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MOLECULAR DETERMINANTS OF ROTAVIRUS VIRULENCE: LOCALIZATION OF A POTENTIAL VIRULENCE SITE IN A MURINE ROTAVIRUS VP4

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Abstract—The molecular basis of pathogenesis *in vivo* for a virulent mouse rotavirus (MRV) and a less virulent bovine rotavirus (BRV) were compared under *in vitro* and *in vivo* conditions. Obvious differences in the mobility of several genomic RNA segments were observed in one-dimensional gels. Under *in vitro* conditions, partial proteolytic peptide mapping identified differences between the two outer capsid proteins of these virus and no difference in inner capsid protein was observed. Since it has been observed by us and others that the gene coding for VP4 protein plays a significant role in determining virulence, the variability observed in the present study between the 84 k proteins (VP4) provided a basis for further investigations in order to locate a potential virulence determinant. A comparison of the carboxypeptidase digests of the MRV- and BRV-VP4 revealed an area of variability between amino acids 307 and 407, which may represent a site of virulence determinant. Under *in vivo* conditions the virulence of both parenteral BRV and MRV isolates and their corresponding reassortants (with replaced gene 4) were studied in murine and bovine hosts. Like their parents, BRV and MRV isolates, reassortants obtained by replacement of gene 4 in BRV with MRV gene 4 indicated that the dose of the virus isolate used and the clinical outcome *in vivo* was determined by gene segment 4. The implications of these findings to elucidate the molecular basis of pathogenesis of rotaviruses are discussed.

Key words: Rotaviruses, reassortants, virulence determinant, pathogenesis, animal models.

Résumé—Les bases moléculaires de la pathogénèse *in vivo* d'un rotavirus murin virulent (MRV) et d'un rotavirus bovin moins virulent (BRV) ont été comparées en conditions *in vitro* et *in vivo*. Des différences manifestes ont été observées en colloïde unidimensionnelle quant à la mobilité de plusieurs segments génomiques d'ARN. En condition *in vitro*, un peptide marqueur partiellement protéolytique a permis d'identifier des différences entre les deux protéines de la capside externe de ces virus mais aucune différence n'a été observée dans la protéine de la capside interne. Depuis que nous avions observé nous-mêmes et d'autres, que le gène codant pour la protéine VP4 jouait un rôle significatif dans la détermination de la virulence, la variabilité observée dans la présente étude entre les protéines 84 k (VP4) constituait une base de recherche pur l'avenir dans le but de localiser un déterminant potentiel de la virulence. Une comparaison de la digestion par la carboxypeptidase de la VP4 du MRV et du BRV a mis en évidence une zone de variabilité entre les acides aminés 307 et 407 qui pourrait bien représenter le site d'un déterminant de la virulence. A été étudiée, chez les hôtes murin et bovin, la virulence des BRV et MRV administrés parentéralement et de virus modifiés correspondants (à qui on a remplacé le gène 4). Comme leurs "parents", les BRV et MRV isolés, les virus modifies obtenus par remplacement du gène 4 du BRV par le gène 4 du MRV, ont mis en évidence que la relation—dose de virus isolé utilisée et la conséquence *in vivo*—était déterminée par le segment génomique 4. Sont ensuite discutées les implications de ces découvertes sur la compréhension du fondement moléculaire de la pathogénèse des rotavirus.

Mots-clefs: Rotavirus, virus modifiés, déterminant de la virulence, pathogénèse, modèles animaux.

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INTRODUCTION

Rotaviruses are the major etiologic agents of acute gastroenteritis in a variety of mammalian neonates [1, 2]. Rotavirus is comprised of a number of structural and non-structural proteins. The major structural proteins includes VP4, VP7 and VP6. The outer capsid of rotaviruses is composed of two proteins, VP7 (38 k) and VP4 (84 k). Both of these proteins have been shown to induce neutralizing antibodies independently [1 and 12a]. Antibodies directed towards the VP7 or VP4 neutralize the virus, inhibit hemaglutination (HA), and passively protect neonatal murine and bovine against rotavirus challenge [12, 12a].

To understand the pathogenesis of the virus, investigations have been underway in order to uncover the molecular mechanisms involved. Basically, all rotaviruses cause the destruction of columnar absorptive cells on the villus tips of the small intestine leading to the development of profuse diarrhoea. This observation helped to formulate the hypothesis that rotaviruses require trypsin for enhancement of infectivity [4–6]. In addition, it pointed to the fact that rotaviruses have a relatively specific cell tropism. The molecular mechanism involved in the latter function has been identified as the cleavage of VP4, at a specific site, to produce two polypeptides fragments VP5* (approximately 60 k) and VP8* (approximately 28 k). Recently it has been demonstrated that monoclonal antibody directed against VP8* can inhibit binding of rotavirus particles to cells *in vitro* [7], indicating that VP8 cleavage product contains an important epitope(s) involved in virus–cell interaction. In addition VP8* portion of VP4 has been found to contain hemagglutinin [8]. We have demonstrated that a peptide corresponding to the trypsin cleavage site (amino acid 232–255) can induce heterotypic protection *in vivo* [9]. Furthermore, pre-treatment of cells with this peptide before infection with rotavirus prevented both the attachment of ^{35}S-labelled virus and plaque formation and intragastric administration of the peptide protected neonatal mice from challenge with virulent rotavirus [10].

In this study, we have attempted to identify virulence determinants in a virulent mouse rotavirus (MRV) and a less virulent bovine rotavirus (BRV) by determining the genetic basis of pathogenicity of these viruses *in vivo* and by comparing the genomic RNA, polypeptides and disassembly conditions *in vitro*.

MATERIALS AND METHODS

Cells and viruses and radiolabelling of virus proteins

Bovine rotavirus isolate C486 and murine rotavirus used in this study were propagated in confluent African green monkey kidney cells (MA-104) as described previously [4]. For radiolabelling the virus was adsorbed to confluent MA-104 monolayers for 1 hr after which time the virus inoculum was replaced with methionine-free MEM. After 3 hr of incubation at 37°C, 25–50 μCi of L-[^{35}S]methionine (Amersham, Oakville, Ontario) per ml was added to the overlay as has been described [11].

Virus purification and extraction of RNA and production of c-DNA probes

Virus purification was achieved using the methods described previously [12]. Virus preparations were initially treated with 1% sodium dodecyl sulfate (SDS) and 100 μg of protease K per ml for 30 min at 37°C. The RNA was then extracted once with 1 vol of phenol and twice with 1 vol of ether and precipitated by the addition of NaCl to a final concentration of 0.15 M and 2 vol in cold 95% ethanol. The RNA was precipitated at

−20°C for 24 hr. Then it was pelleted at 5500 *g* for 30 min in a Sorvall SP-600 rotor and resuspended in 500 μl of TNE (0.1 M Tris–hydrochloride [pH 7.8], 0.15 M NaCl, 0.001 M EDTA). The amount and purity of the RNA was determined by spectrophotometry at wavelengths of 260 and 280 nm.

Complementary DNA probes of the genomic RNA of MRV and BRV were made by incubating 1.0 μg of heat-denatured double-stranded genomic RNA resuspended in 20 μl 5× random primer buffer (0.25 M Tris–HCl [pH 8.1], 10 mM dithiothreitol, 25 mM $MgCl_2$, 0.2 M KCl), 2 mM solution of each unlabeled dNTP (New England Biolabs), 100 μCi [^{35}P]dNTP (Amersham), 200 units reverse transcriptase (New England Biolabs) at 37°C for 1 hr. Then, 2 μl of 0.5 M EDTA and 12 μl of 3 M NaOH were added and further incubated for 12 hr at 37°C. Separation of labeled DNA from unincorporated dNTPs was done by chromatography through a column of Sephadex G-50 (Pharmacia, Uppsala, Sweden).

Polyacrylamide gel analysis of proteins and RNA

Virus proteins and RNA were examined by polyacrylamide gel electrophoresis as has been described by Laemmli [13]. Protein samples for electrophoresis were processed as has been described earlier [14].

Peptide mapping (Cleveland digest)

Partial proteolytic digestion of the virus protein was performed according to the procedure of Cleveland *et al.* [15]. A 5%–5 cm stacking gel and a 12.5% or 15%–11 cm resolving gel were employed for the digestion and resolution of digest. The resulting Cleveland digests were then electroblotted to nitrocellulose paper as described below and reacted with antibodies.

Electroblotting of proteins and RNA

Proteins were transferred from polyacrylamide gels to 0.45 μm nitrocellulose paper as has been described by Towbin *et al.* [16]. After transfer, either the nitrocellulose strips were stained if non-radiolabeled samples were used or exposed to 3 M film (Picker International, Saskatoon, Saskatchewan, Canada) if radiolabeled samples were used. Nitrocellulose paper was also subjected to the immunoblot–ELISA.

Genomic RNA was transferred from polyacrylamide gels to gene screen (New England Nuclear, Lachine, Quebec, Canada) via electroblotting for 4 hr at 4°C in phosphate buffer at 1 mA. After transfer, the gene screen was subjected to hybridization.

Immunoblot–ELISA

Rotavirus-specific polypeptides were detected by the immunoblotting technique as described by Towbin *et al.* [16]. The procedure for reacting antisera with polypeptides transferred to nitrocellulose as has been described by Braun *et al.* [17]. The primary antibody used was either a monoclonal antibody directed against VP7 (amino acid 275–295) [11] or to the trypsin-cleavage site on VP4 (amino acid 232–255) [12b, 18].

Hybridization of cDNA probes to genomic RNA immobilized on filters

The gene screen filters carrying genomic RNA were prehybridized in 10–25 ml of 4 × SSC, 10 × Denhart, 0.1% SDS in a sealed bag at 65°C. Prehybridization mix was discarded after 1 hr and 10–25 ml of hybridization mix was added (4 × SSC, 1 × Denhart, 0.1% SDS, 50–100 μg/ml salmon sperm DNA) with ^{32}P-labelled cDNA (about 1 × 10^6 cpm

Cerenkov/filter). Hybridizations were done at 65°C until the background of radioactivity was reached in the wash. The filters were then dried and autoradiographed with intensifying screens at −70°C. The degree of hybridization of total genomic MRV-cDNA and total genomic BRV-cDNA to the genomic profile of BRV was determined by scanning the autoradiographs with a densitometer and then comparing the areas under corresponding peaks representing individual RNA segments.

Generation and characterization of reassortants

Reassortant viruses were derived by co-infection of MA-104 cells with BRV and MRV isolates of rotaviruses at a multiplicity of 5.0 as previously described [19]. After 48 hr of incubation at 37°C, cultures infected with BRV and MRV were harvested by freezing and thawing. Progeny virus was titrated by plaque assay as previously described [20]. Individual progeny plaques were picked, passaged twice in MA-104 cells, and characterized by analysis of reassortant viruses by SDS–PAGE and RNA-cDNA hybridization as described above.

In vivo experimental design and sampling procedure

In case of murine model, neonates, 7-days old, were used in all experiments. Each group of five neonatal mice were inoculated intragastrally with either rotavirus isolates or their reassortants (2×10^3). The virus preparations were administered orally as described by [12]. Virus infection was evaluated clinically 24 hr postinoculation (HPI) and scored as follows: no sign of the disease (−) and maximum clinical score (+ + + +).

In case of bovine, a gut loop technique was used. Basically, this approach involved surgical removal of the jejunum from a gnotobiotic calf under anaesthesia, and then segmentation by tying off every 8–10 cm. Virus was then inoculated into sections of the jejunum along with appropriate controls in adjacent sections. The volume of resting fluid in each section of the jejunum, after a 24-hr period, indicated the severity of the infection [21, 22].

RESULTS

Comparison of virulence between mouse rotavirus (MRV) and bovine rotavirus (BRV) in vivo and in vitro

As shown in Table 1, MRV was found to be more virulent than BRV. Table 2 also illustrates that even in the bovine system, the MRV (cell-culture adapted murine rotavirus isolate) is more virulent. It induces 1–1.7 ml of fluid as compared to undetectable fluid levels in segments inoculated with the BRV (cell-culture adapted bovine rotavirus isolate).

Comparison of the kinetics of replication of MRV and BRV *in vitro* supports the *in vivo* findings. Such that no BRV proteins are identifiable at 4 hr post-infection, however, in the

Table 1. Diarrheal score in 7-day old neonatal mice inoculated with reassorted gene 4-BRV or -MRV

Control	MRV	MRV with BRV gene 4	BRV	BRV with MRV gene 4
−	+ + + +	+	+	+ + + +

Each group of five neonatal mice were inoculated intragastrally with either rotavirus isolates or their reassortants (2×10^3) generated and characterized as described in the Materials and Methods section. 24 HPI diarrhea was scored as reported earlier [12]. No sign of the disease (−) and maximum clinical score (+ + + +).

Table 2. Virulence of MRV and BRV in calf intestinal loops*

Amount of fluid in jejunum (ml)			
MRV		BRV	
Mid-jejunum	Low-jejunum	Mid-jejunum	Low-jejunum
1.7	1.0	0	0

*The intestinal loops of calves were produced under anesthesia [21, 22]. These loops were inoculated with 5.0 ml of either MRV or BRV suspension (2×10^6) or mock-infected with MEM only. Intestinal loops were returned back into abdominal cavity and the cavity closed by sutures and was re-opened 24 HPI to assess the affect produced by the isolates as described in Materials and Methods section. No fluid was obtained from the mock-infected loops.

case of MRV several virus-specific proteins are already present at this time (data not shown).

Molecular characterization of MRV and BRV

The proteins and genomic profile of BRV and MRV were compared using PAGE, for both viruses, gene segments 1, 3, 4, 5, 10 and 11 were migrating at different rates. When protein profiles of MRV and BRV were examined we could only observe the difference is in the mobility of VP4. When the protein profile were compared following their digestion using different enzymes, both BRV and MRV digest patterns of the 110 k (VP1), 92 k (VP2) and 45 k (VP6) inner proteins indicate that they are identical (data not shown). The outer shell proteins, however, appeared to be different when their digest patterns were compared (Fig. 1). Specifically, the reduced forms of MRV- and BRV-VP7 glycoproteins exhibit a different chymotrypsin (Panel 1, lanes A and B) *S.* aureus V8 protease (Fig. 1, Panel 1, lanes D and E) and papain (Fig. 1, Panel 1, lanes G and H) digest patterns. The reduced forms of VP4 also exhibit different papain and *S. aureus* V8 protease digest patterns (Fig. 1, Panel 2).

Reactivity of MRV and BRV proteins with monoclonal antibodies directed to important biological sites

As illustrated in Fig. 2, the monoclonal antibodies directed against conserved sites in two outer capsid proteins i.e. VP7 (amino acid 275–295) and VP4 (amino acid 232–255) reacted equally well with both corresponding MRV-glycoproteins, suggesting that this site was not significantly altered. Lanes C, D, and corresponding immunoblots C′ and D′ illustrate that this antiserum also detects this site in the MRV-VP4.

Behavior of BRV and MRV in the presence of calcium chelating and chaotropic agents

Figure 3A (lanes 1, 2) shows that 50 mM EDTA–0.01 M Tris–HCl pH 7.4 can be used to remove most of the VP4 glycoprotein from the outer capsid of BRV. Under these conditions, most of the virus is converted to the single-shelled form. When MRV was incubated under the same conditions, very little glycoprotein was removed from the particles. However, if MRV was incubated in 50 mM EDTA–0.01 M Tris–HCl, pH 8.0, instead of pH 7.4, most of the glycoproteins were removed (Fig. 3A, lane 3–4). $CaCl_2$ treatment of EDTA-treated BRV solubilized nucleocapsid protein at both pH levels (Fig. 3B, lane 2). These preparations contain little or no contaminating capsid proteins. In contrast, $CaCl_2$ treatment of MRV at either pH (7.4 or 8.0) level solubilized very little nucleocapsid protein and these samples generally contained mostly glycoprotein (Fig. 3B,

lanes 1, 3–6). Comparison of supernatants from EDTA and $CaCl_2$ treatments (lanes 5 and 6) of MRV also confirms that glycoprotein is solubilized by both reagents.

Identification of a potential virulence site

Reassortants between MRV and BRV, where only gene 4 was exchanged, exhibited a pattern of virulence corresponding to the parent from which the gene 4 was derived (Table 1). The digestive profiles of BRV- and MRV-VP4 (digested with carboxypeptidase A) were compared by scanning the autoradiographs and then matching the peaks which represent peptide bands (Fig. 4). A comparison of peptide fragments indicates that there is a variation in the banding pattern between MRV and BRV peptides in the molecular weight range 43–53 k, a difference located between amino acid 307 and 407 of MRV-VP4.

DISCUSSION

VP4 was chosen as the most likely candidate protein for carrying out a virulence determinant since it was reported by Offit *et al.* [19] that the gene coding for this protein plays a major role in conferring virulence on the virus. In addition *in vivo* studies using BRV with replaced gene 4 from MRV confirmed this [12, 23] (Tables 1 and 2). Analysis of BRV- and MRV-VP4 by carboxypeptidase digestion revealed only one region of heterogeneity between these two viruses in the area spanning amino acids 307 and 407. However, it should be pointed out that the difference between the two isolates can not only be in the area spanning amino acid 307 and 407 and that the analysis of sequence data on gene 4 of both isolates might reveal other site(s) as well. Therefore, confirmation of the differences in potential virulence sites located on gene 4 of both isolates will have to wait till MRV-gene 4 sequence data becomes available. For now, it appears that there are at least two factors which may account for the increased virulence of MRV. The first one is the increased stability of MRV, which may be a consequence of the observed differences in the outer shell proteins of MRV when compared to BRV [33, 34, 37, 38]. The second factor is a specific difference in the primary structure of VP4 which may provide the molecular basis for the increased stability and consequently the increased virulence. Alternatively, the stability and virulence determinant may define two different strategies for conferring virulence on rotaviruses. The MRV has also been shown to be extremely stable in the environment [24] as compared to other human and animal rotavirus isolates explaining the difficulty in keeping rodent's colonies rotavirus-free and preventing nosocomial rotavirus infection [20, 25–29].

Several *in vitro* and *in vivo* experiments carried out on different rotavirus isolates have shown that gene 4 is an important virulence factor in the pathogenesis of rotavirus [1, 19, 30]. In spite of these studies, the subject is still open for controversy. Recently, Broome *et al.* [31] have reported that VP4 as well as the other two major rotavirus proteins, VP6 and VP7 are not associated with virulence. However, they did not provide sufficient experimental evidence to demonstrate which other gene(s) product(s) are involved in virulence determination. Based on their limited experimental data they have speculated that either gene 5 and/or 2 may be involved in rotavirus virulence determination. It will be interesting to see what data they obtain in their proposed follow up studies.

In support of our findings, virulence studies carried out by Offit *et al.* [19] using heterologous rotavirus in homologous host (murine model) observed that virulence is determined by gene 4. Furthermore during the comparative analysis of the VP4 gene of

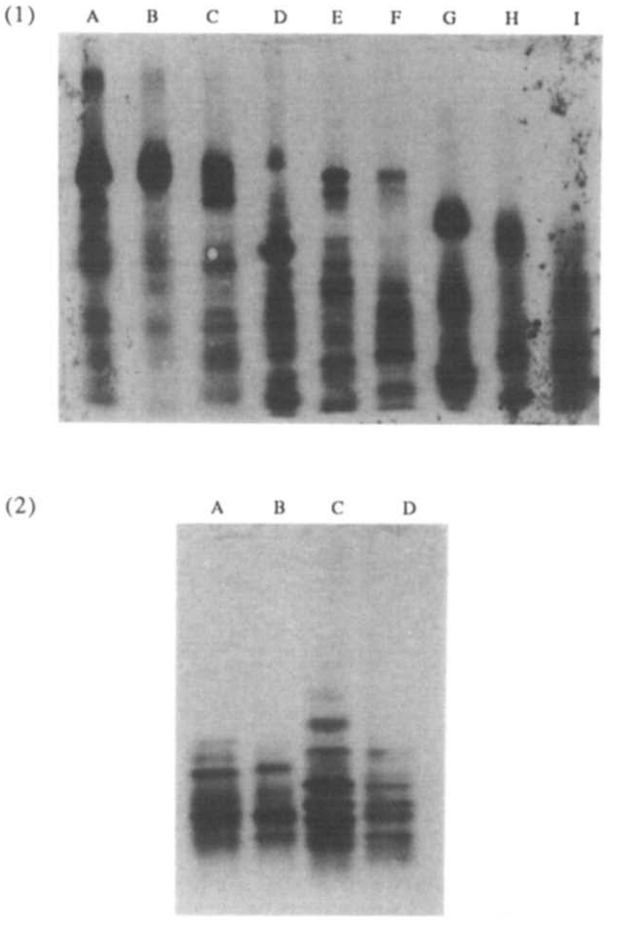

Fig. 1. Partial proteolytic digests of the outer shell proteins of the MRV and BRV. Panel 1 illustrates the digest pattern BRV-VP7 in its reduced form (lanes A, D, G), the MRV-VP7 in its reduced form (lanes B, E, H) and the MRV-VP7 in its unreduced form (C, F, I). Lanes A–C represent digestion patterns generated after exposure to 5.0 μg chymotrypsin, lanes D–F represent digestion patterns generated after exposure to 20 μg *S. aureus* V8 protease, lanes G–I represent digestion patterns generated after exposure to 50 μg papain. Panel 2 illustrates the digest pattern of BRV-VP4 in its reduced form (lanes A and C) and MRV-VP4 in its reduced form (lane B and D). Lanes A and B represent digestion patterns generated after exposure to 10 μg papain, lanes C and D represent digestion patterns generated after exposure to 5.0 μg *S. aureus* V8 protease.

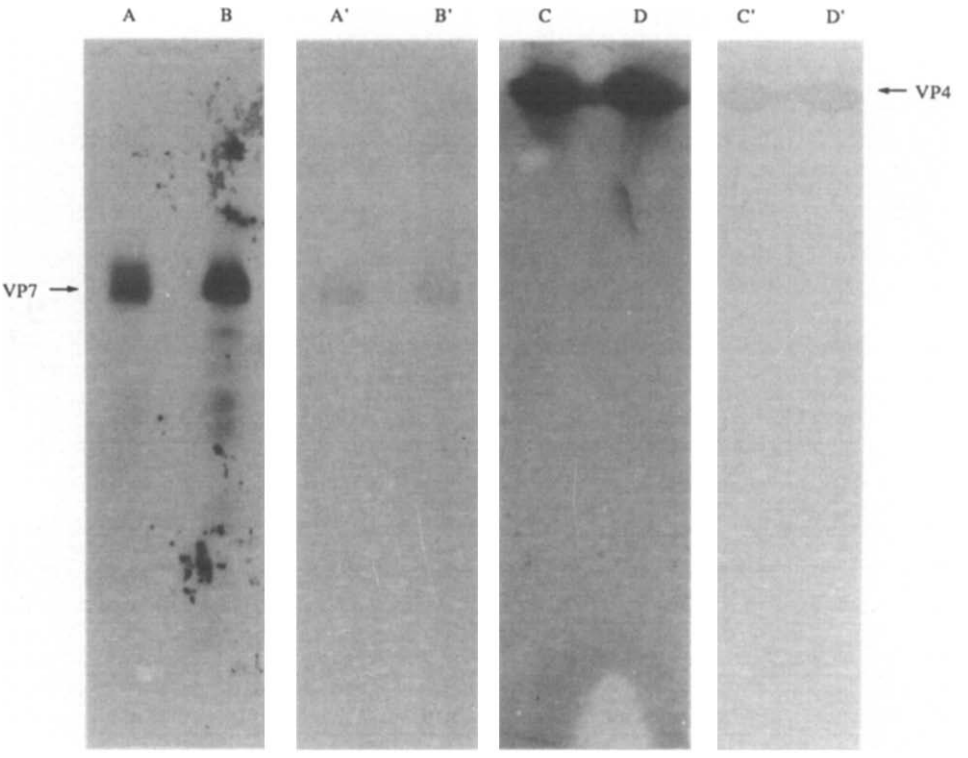

Fig. 2. The reactivity of MRV and BRV outer shell proteins VP7 and VP4 with specific monoclonal antibodies. Lanes A and B represent autoradiographs of the MRV- and BRV-VP7 respectively, electrophoresed on a 12.5% polyacrylamide gel. Lanes A′ and B′ represent the corresponding immunoblot reactions with a monoclonal antibody directed against VP7 (amino acid 275–295). Lanes C and D represent autoradiographs of the MRV- and BRV-VP4 respectively, electrophoresed on a 15% polyacrylamide gel. Lanes C′ and D′ represent the corresponding immunoblot reactions with a monoclonal antibody specific for VP4 trypsin-cleavage site (amino acid 232–255).

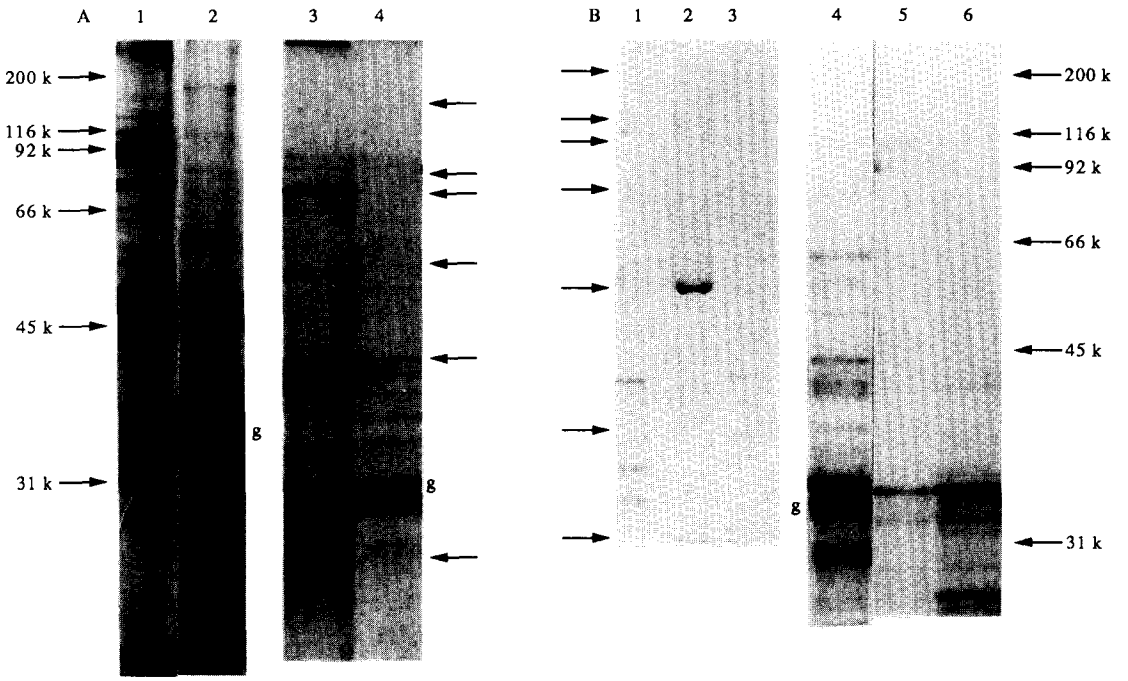

Fig. 3. Polyacrylamide gel electrophoresis showing the effects of EDTA and $CaCl_2$ treatment on BRV and MRV. (A) BRV and MRV were incubated in 50 mM EDTA–0.01 M Tris–HCl pH 7.4 or pH 8.0, respectively, at 4°C for 30 min. Subviral and undegraded particles were separated from solubilized protein by ultracentrifugation. BRV, pellet (lane 1); BRV, supernatant (lane 2); MRV, pellet (lane 3); MRV, supernatant (lane 4). (B) EDTA-treated particles were incubated in 1.5 M $CaCl_2$–0.01 M Tris–HCl, pH 7.4 at 20°C for 20–30 min, undegraded particles and cores were removed from solubilized protein by ultracentrifugation. Solubilized MRV proteins are shown in lanes 1, 3, 5 and 6. Solubilized BRV protein is in lane 2. For comparison, MRV polypeptides solubilized by EDTA treatment are shown in lane 4. The position of molecular weight markers are indicated by the arrows for each set of gels. VP6 is located at the 45 k position, whereas VP7 glycoprotein is denoted "g" for each gel.

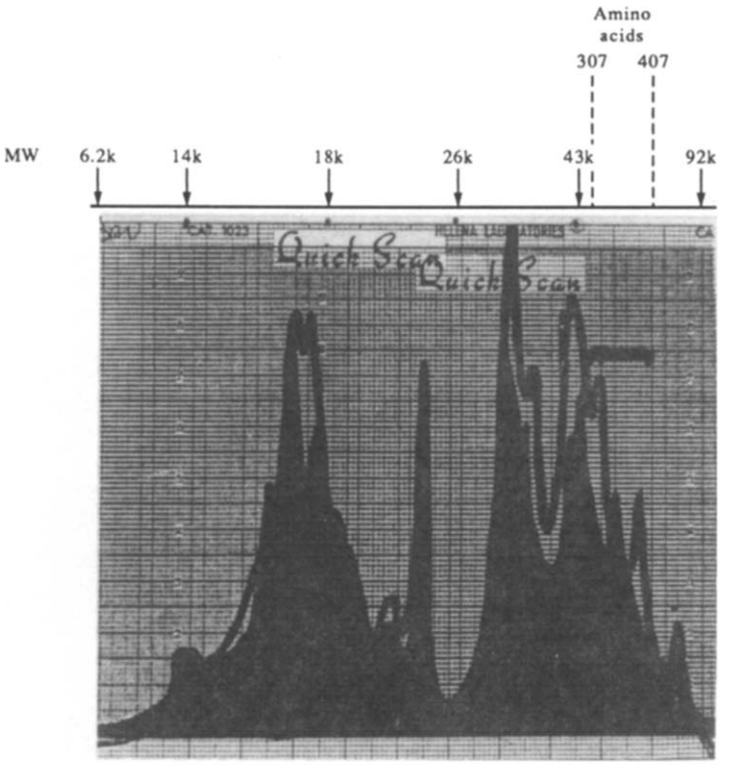

Fig. 4. Comparison of carboxypeptidase digests between MRV- and BRV-VP4. MRV- and BRV-VP4 were subjected to proteolytic cleavage according to the procedure described in the Materials and Methods section. The autoradiograph of the digest patterns was scanned using a densitometer. The scans of the MRV-VP4 (solid area) and of the BRV-VP4 (shaded area) were superimposed and the location of peaks compared. Variation between peaks is marked with a bracket and corresponds to molecular weight positions of between 43 and 53 k. This localizes the area of variability between amino acids 307–407.

different rotaviruses Nishikawa *et al.* [32] observed that the VP4 of standard strain of Nebraska calf diarrhoea virus (NCDV) which is virulent for cows differed in only one amino acid (position 23, Gln to Lys) from the VP4 of an NCDV mutant which was attenuated both for cows and for children which further underline the role played by gene 4 in determining rotavirus virulence. In our studies, however, both of the rotavirus isolates used, replicate very well in the murine system and virus isolated from neonatal mice can be assayed *in vitro* in cell culture [12]. Furthermore, our reassortants where only gene 4 was replaced, exhibited a pattern of virulence corresponding to the parents from which the gene 4 was derived (Table 1).

Further studies are needed in order to identify more precisely the region responsible for conferring virulence. One of the approaches being used is to synthesize peptides corresponding to regions between amino acids 307–407 and then using them to raise antibodies. Antibodies produced in this manner will then be tested for their ability to reduce the virulence of a particular rotavirus. In addition, the genes coding VP4 from virulent and less virulent rotaviruses are being sequenced to more specifically localize the region(s) of variability.

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