VP7 Serotype-specific Glycoprotein of OSU Porcine Rotavirus: Coding Assignment and Gene Sequence

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(Accepted 9 July 1986)

SUMMARY

With a reassortant from a cross of human rotavirus DS-1 (serotype 2) and OSU (serotype 5) it was determined that the OSU major neutralization glycoprotein antigen (VP7) was encoded by gene segment 9. A full-sized cloned cDNA copy of the OSU gene 9 was produced and sequenced. Hybridization of such labelled cDNA with the corresponding segment of a reassortant DS-1 × OSU virus confirmed the coding assignment. Comparison of the deduced amino acid sequence of the VP7 of OSU with those previously determined for five other rotavirus strains, representing four distinct serotypes, revealed some hydrophilic regions that exhibited significant homology and other hydrophilic domains with greater amino acid divergence. In one of the latter hydrophilic domains each of the five serotypes had a distinct amino acid substitution at residue 146, suggesting that it may be involved in serotype specificity.

INTRODUCTION

Rotaviruses are the most common aetiological agents of acute non-bacterial gastroenteritis in humans as well as in a variety of animal species (Flewett & Woode, 1978; Kapikian et al., 1980). Their genome consists of 11 segments of dsRNA with sizes ranging from 637 to 3500 base pairs (Gorziglia et al., 1983; Rixon et al., 1984). The genomic dsRNA is enclosed within a double-layered protein capsid formed by at least five structural polypeptides (Novo & Esparza, 1981; Ericson et al., 1982). Two proteins present on the outer capsid of rotaviruses, VP3 and VP7, elicit antibodies capable of neutralizing virus infectivity (Greenberg et al., 1983a, b; Killen & Dimmock, 1982; Matsuno & Inouye, 1983). Recently, Hoshino et al. (1985) have demonstrated that both proteins are equally immunogenic. The outer capsid glycoprotein, VP7, with an apparent mol. wt. of 38K (Ericson et al., 1983) is coded for by genomic segment 8 or 9. The gene coding assignment for this glycoprotein has been demonstrated in three different ways: (i) in vitro translation of an individual genomic dsRNA or ssRNA transcript corresponding to segment 8 (UK calf rotavirus, serotype 6) or segment 9 (SA11 simian rotavirus, serotype 3) yielded VP7 as its product (Both et al., 1983; Mason et al., 1980; McCrae & McCorquodale, 1982a), (ii) Northern blot hybridization indicated that RNA segment 8 (HU/5 human rotavirus, serotype 2) encodes the major outer shell neutralization glycoprotein (Dyall-Smith & Holmes, 1984), and (iii) analysis of reassortant rotaviruses exhibiting different neutralization specificities

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established that, depending upon the virus strain, VP7 is encoded by either genome segment 9 (W human rotavirus, serotype 1) or segment 8 (DS-1 human rotavirus, serotype 2) (Greenberg et al., 1983a).

Serotypes of rotavirus are defined by neutralization of viral infectivity and to date seven serotypes have been identified, four of which (serotypes 1 to 4) are found in humans and five (serotypes 3 to 7) in animals. Two different serotypes of rotavirus have been identified in pigs (Bohl et al., 1984). One serotype (porcine serotype 1) includes the OSU, EE, and A-580 strains, with OSU serving as the prototype virus, while the other serotype (porcine serotype 2) includes the Gottfried and SB-5 strains with Gottfried serving as the prototype virus. These antigenic differences were confirmed by Hoshino et al. (1984) who classified the Gottfried strain as serotype 4 and OSU as serotype 5 in a unified human, animal and avian rotavirus numbering system.

In the present study, we used genetic reassortment to identify the gene of OSU that encodes the VP7 glycoprotein and used hybridization techniques to confirm this coding assignment. Molecular cloning of dsRNA virus genome segments has been described, including the production of cDNA clones from several human rotaviruses (Gorziglia et al., 1983; Imai et al., 1983), a bovine strain, UK (McCrae & McCorquodale, 1982b), and a simian strain, SA11 (Both et al., 1982). We have cloned and sequenced the gene of porcine rotavirus strain OSU which codes for VP7 and compared this gene and its deduced amino acid sequence with the corresponding genes of human rotaviruses Wa and HU/5 (Dyall-Smith & Holmes, 1984; Richardson et al., 1984), bovine rotaviruses UK and NCDV (Elleman et al., 1983; Glass et al., 1985) and simian rotavirus SA11 (Arias et al., 1984; Both et al., 1983) in an attempt to identify regions of the VP7 gene responsible for serotype specificity as well as conserved antigenic sites.

**METHODS**

Genetic reassortment, selection and genotyping of reassortants and plaque reduction neutralization assay. MA-104 cell monolayers were infected with the DS-1 (serotype 2) strain of human rotavirus at a m.o.i. of approximately 1 and incubated for 1 h at 37 °C. The monolayers were then superinfected with porcine rotavirus OSU (serotype 3) at a m.o.i. of approximately 1 and the cultures were incubated for another hour at 37 °C. The cell monolayers were washed twice and fed with Eagle’s MEM. When 50 to 80% of the cells showed c.p.e. the cultures were then frozen and thawed once and harvested. The harvest from co-infected cultures was inoculated onto MA-104 cell monolayers in the presence of hyperimmune guinea-pig antiserum raised against the SB-1LA strain of porcine rotavirus. This rotavirus was shown to have two distinct neutralization specificities, serotype 5 based on VP3 (the fourth gene function) and serotype 4 based on VP7 (the ninth gene function) (Hoshino et al., 1986). Each reassortant was plaque-purified three times in MA-104 cell monolayers. RNA–RNA hybridization assays were performed to determine the parental origin of each of the genes present in each reassortant rotavirus as described previously (Flores et al., 1982). Serotype characterization of each reassortant was performed by plaque reduction neutralization assay as previously described (Hoshino et al., 1982; Wyatt et al., 1982).

Isolation of dsRNA. The OSU strain was obtained in its 37th passage in MA-104 cell monolayers from E. H. Bohl (Ohio Agricultural Research and Development Center, Wooster, Ohio, U.S.A.). Virus suspensions were prepared by infecting confluent MA-104 cell monolayers with phosphate-buffered saline (PBS) with virus suspended in 10 μg/ml trypsin. After 1 h adsorption at 37 °C, cells were washed once with PBS; Eagle’s MEM containing 1 μg/ml trypsin was then added to the cultures. Infected cells were incubated at 37 °C until complete c.p.e. was observed. Virus was purified by Freon-113 extraction of infected cells, followed by pelleting through a cushion of 45% sucrose (Novo & Esparza, 1981). The virus pellet was resuspended in a buffer containing 20 mM-Tris–HCl pH 7.4 and 15 mM-EDTA and incubated at 37 °C for 2 h. Single capsid virus was purified by isopycnic centrifugation in a CsCl gradient. For extraction of viral RNAs, purified particles were resuspended in 20 mM-Tris–HCl pH 7.4 containing 1% SDS and incubated at 55 °C for 1 h, followed by phenol–chloroform extraction and ethanol precipitation in the presence of 0.3 M-sodium acetate.

Synthesis and cloning of OSU rotavirus cDNA. The strategy used to clone OSU rotavirus genes has been previously described (Gorziglia et al., 1983). dsDNA was melted by incubation at 50 °C for 45 min in 90% DMSO and the 3' ends of both strands were tailed with poly(A) using Escherichia coli poly(A) polymerase. The polyadenylated RNA was purified by chromatography on oligo(dT)–cellulose and reverse-transcribed after priming with oligo(dT). After removal of the RNA template by alkaline treatment, cDNA strands were purified by filtration through a Sephadex G-75 column. Fractions containing cDNA were pooled, and lyophilized. The 3' ends were then tailed with oligo(dC) and sized by preparative alkaline agarose gel electrophoresis. SsDNAs eluted from the gel were then allowed to hybridize. Double-stranded cDNAs were inserted into the PstI site of pBR322 by
Fig. 1. Analysis of genomes of human rotavirus strain DS-1 × porcine rotavirus strain OSU reassortants, 10-1 and 13-1. Genomic RNAs of (a) 10-1, (b) DS-1, (c) 13-1 and (d) OSU were hybridized to single-stranded $^{32}$P-labelled mRNA transcripts of OSU. In 10-1 only the 9th gene in the 7, 8, 9 complex is derived from OSU, and in reassortant 13-1 only the 8th gene in the 7, 8, 9 complex is derived from OSU. Note the absence of homology between the DS-1 genomic RNAs and the OSU probe.

dC/dG tailing. The hybrid plasmids were used to transform E. coli HB101 cells and transformants containing recombinant plasmids were identified by growth in tetracycline-containing medium. The sizes of the inserts were determined by rapid lysis of transformants followed by agarose gel electrophoresis and by electrophoresis of PstI-excised inserts.

Nucleic acid hybridization. Double-stranded RNAs from reassortant or parental rotaviruses were extracted from concentrated virions with 1% SDS, followed by phenol–chloroform extraction and precipitation with ethanol in the presence of sodium acetate (0.2 M). Electrophoresis of dsRNAs was performed on 6% polyacrylamide gels with 3% stacking gels in a discontinuous Tris–glycine buffer system as described by Laemmli (1970). After electrophoresis, the dsRNA bands were stained with ethidium bromide and visualized under u.v. light. The RNAs were electrotransferred to diazotized-aminophenylthioether (APT) paper and used for hybridization with $^{32}$P-labelled nick-translated plasmids carrying an insert of 1100 bases (clone p42A) as previously described (Cashdollar et al., 1982). To confirm that clone p42A contained a full-length cDNA copy corresponding to the VP7 gene, a dot hybridization assay (Flores et al., 1983) was performed using two $^{32}$P-labelled oligonucleotide probes which corresponded to the 5' and 3' ends of the NCDV gene encoding VP7 (Glass et al., 1985).

Sequence determination. Recombinant plasmids were prepared from cleared lysates of transformant bacteria (Birnboim & Doly, 1979). Restriction fragments of the insert were isolated from polyacrylamide gel by crushing and eluting in 0.5 M-ammonium acetate, 1 mM-EDTA pH 8.0 and centrifugation through a disposable microfilter unit (CENTREX, Schleicher & Schuell). Sequencing was performed using the chemical method (Maxam & Gilbert, 1977). Both the positive and the negative strand fragments were end-labelled and the gene was read in full in both directions. The proteins encoded by these nucleotide sequences were examined by computer analysis (Kyte & Doolittle, 1982).

RESULTS

Generation of human (DS-1 strain) × porcine (OSU strain) reassortants, genotyping by RNA–RNA hybridization and serotypic characterization by plaque reduction neutralization assay

Under the selective pressure of hyperimmune serum raised against the porcine SB-1A virus, two different reassortants were selected and used in this study. One reassortant (10-1) derived nine genes (including the 9th) from porcine OSU and two (the seventh and eighth genes) from human DS-1. The other reassortant (13-1) derived eight genes (including the 8th) gene from OSU and three (the fourth, seventh and ninth genes) from DS-1 (Fig. 1).
Fig. 2. Identification of the cloned rotavirus VP7 gene. Lanes 1, DS-1; lanes 2, 10-1; lanes 3, OSU. (a) Autoradiogram showing that the labelled probe hybridized to OSU dsRNA segment 9 and the reassortant (10-1) which contained only the 9th gene of OSU in its 7-8-9 gene complex. (b) Total rotavirus dsRNAs of DS-1, 10-1 and OSU were electrophoresed in a 6% polyacrylamide gel using the buffer system described by Laemmli (1970), stained with ethidium bromide, and photographed under u.v. light. The RNA was transferred to diazotized-APT paper and hybridized to the 32P-labelled nick-translated plasmid carrying the VP7 gene identified in (a).

Hyperimmune antiserum to porcine OSU virus neutralized the DS-1 × OSU reassortant 10-1 to a high titre (>1:81920) whereas hyperimmune antiserum to human DS-1 virus did not neutralize the reassortant virus (<1:80), providing evidence that the ninth gene of OSU codes for the major neutralization protein VP7. Reassortant 13-1 was neutralized to a high titre by hyperimmune antiserum to DS-1 (1:20480), but not by hyperimmune antiserum to OSU (<1:80), indicating that the eighth gene of OSU does not encode the VP7 protein.

Cloning of double-stranded cDNA

Oligo(dC)-tailed cDNAs migrated in alkaline gels similarly to the original cDNAs. Several discrete bands of cDNA with sizes ranging from 690 to 3500 bp were identified; these included bands of approximately 1100 bp that could contain copies of genes 7, 8 and 9. Double-stranded cDNAs corresponding to these segments were annealed with oligo(dG)-tailed pBR322 and each mixture of hybrid plasmids was used to transform four different preparations of competent E. coli HB101 cells. Transformed cells were selected overnight in LB broth containing 15 mg tetracycline per ml. The plasmid DNA of the tetracycline-resistant cells was isolated by rapid lysis and the PstI-excised inserts were analysed in agarose gels. Two discrete bands were found to correspond to inserts of approximately 1100 and 1150 bp respectively (data not shown). A hybrid plasmid containing a cDNA copy of VP7 from NCDV (Glass et al., 1985) was 32P-labelled by nick translation and used as a probe to identify clones containing the VP7 gene. A positive clone, p42A, was isolated.

Coding assignment by gene hybridization

The plasmid p42A was 32P-labelled by nick translation and used for hybridization with genomic dsRNA segments immobilized on APT paper (Fig. 2). Plasmid p42A hybridized with
OSU rotavirus VP7 gene

<table>
<thead>
<tr>
<th>Genome Segment</th>
<th>Nucleotide Sequence</th>
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<tr>
<td>OSU</td>
<td>ATTGCACTGCAAGTTTGCTGATACTGCTTATTTGAATGAAATATCCAGTTCAGAGGTTTTGTTGTTG</td>
</tr>
<tr>
<td>DS-1</td>
<td>ATGTGCTGTAATATTCATTTAATTGGAAGAGATGGTGGCAAGTTTCATTATTTAATTTCTACAGTTTTCATGTTAT</td>
</tr>
<tr>
<td>NCDV</td>
<td>CATGTCTGAATTGGGTTTAATATTTGGAATGGCTATGTAATCCAATGGATATAATGCTATATCATCATCAGCAAACAGATGAAGCTAATATACTCATGCTGAAGTATTTATTTGG</td>
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Fig. 3. Comparison of nucleotide sequence of the VP7 gene of OSU with homologous genes from human Wa (Richardson et al., 1984), human HU-5 (Dyall-Smith & Holmes, 1984), simian SA11 (Both et al., 1983), bovine UK (Elleman et al., 1983) and calf NCDV (Glass et al., 1985). Their serotypes are given in parentheses (top left corner). Underlined bases indicate the positions of the two initiation codons and the single termination codon.

The cloned OSU gene 9 is similar to five previously sequenced VP7 genes in that it is 1062 nucleotides long with two, in-phase, AUG triplets at nucleotides 49 to 51 and 136 to 138. The TAG codon at nucleotides 1027 to 1029 terminates the open reading frame in each of the genes.

VP7 gene and deduced protein sequences

The first ten nucleotides at the 5’ end and the last nine nucleotides at the 3’ end are conserved in the VP7 genes of all rotavirus strains sequenced thus far (Fig. 3). Oligodeoxynucleotide probes corresponding to the first 18 bp (5’ end) and the last 15 bp (3’ end) of the NCDV gene 9 sequence were used to confirm by dot hybridization that the clone p42A contained a full-length cDNA copy of gene 9 (data not shown).

The genome segment 9 of OSU and with the gene segment corresponding to OSU gene 9 of the reassortant rotavirus (10-1). In contrast, DS-1 segment 9 did not hybridize with plasmid p42A.
Fig. 4. Comparison of the amino acid sequences of the VP7 of six different rotaviruses representing five different serotypes (in parentheses). Boxes A to G are regions having three or more amino acid substitutions clustered within a frame of four to 15 amino acids. Shaded areas mark possible carbohydrate attachment sites.

is present in all strains except NCDV (Fig. 4). The remaining strains have two or three potential glycosylation sites on their VP7. Wa, HU/5, UK and NCDV have a common site at amino acid residues 238 to 240. NCDV and UK share a site at amino acid residues 318 to 320 and HU/5 has a distinctive site at amino acid residues 146 to 148. Eight cysteine residues are conserved in all strains although SA11 has an additional cysteine at position 32. It is interesting to note that all the cysteine residues are located outside the regions where there is most divergence of amino acid sequence (Fig. 4).

There are seven regions (A to G) in the protein sequence which exhibit the greatest variability, containing three or more substitutions within a four to 15 amino acid frame. At residue number 146, there is a distinct amino acid substitution for each of the five serotypes while two strains of the same serotype have a conserved sequence. The seven regions (A to G) of OSU were compared in a hydropathicity profile to the five previously sequenced strains (Fig. 5). In general, the hydropathicity profiles of the six strains were remarkably similar and characterized by a long hydrophobic region at the N terminus (amino acid residues 1 to 48) and a short common hydrophilic area at the C terminus. Three of the regions (A, D and F) were in hydrophobic domains and four (B, C, E and G) were in hydrophilic domains. The latter domains may represent potential sites for serotype specificity. Three other hydrophilic domains at amino acid residues 170 to 181, 285 to 293 and 310 to 320, common to all strains, are outside the regions of variability and may represent areas of common antigenic determinants.

**DISCUSSION**

Because the 7th, 8th and 9th gene segments of porcine rotavirus strain OSU have a similar pattern of migration under a variety of electrophoretic conditions, the coding assignment for the type-specific glycoprotein VP7 was determined using reassortants between human rotavirus DS-1 and the porcine OSU strain. DS-1 was chosen for this purpose because it is not significantly related by cross-neutralization to rotavirus strains belonging to other serotypes (Hoshino et al., 1984). Absence of significant homology between OSU and DS-1 was confirmed by failure of 32P-labelled ssRNA transcripts from OSU to anneal to double-stranded genomic RNAs of DS-1. Reassortants between DS-1 and OSU were selected under the pressure of hyperimmune antiserum raised against the SB-1A strain. This serum neutralized the OSU strain
Fig. 5. Comparison of hydrophobic and hydrophilic regions of the VP7 glycoproteins of five different serotypes of rotaviruses by the method of Kyte & Doolittle (1982). Locations of amino acid substitutions with respect to OSU are shown at the base of the figure. Letters A to G indicate the regions of variation in Fig. 4.

because both viruses are related by VP3 but not by VP7 (Hoshino et al., 1986). Two reassortants were selected, one (10-1) with genes 7 and 9 from DS-1 and all other genes from OSU, and the other (13-1) with genes 4, 7 and 9 from DS-1 and all other genes from OSU. Neutralization assays, RNA–RNA and RNA–DNA hybridizations were used to assign the OSU VP7 glycoprotein to genome segment 9.

Segment 9 has the potential to code for two proteins depending on whether protein synthesis is initiated at the first (nucleotides 49 to 51) or second AUG (nucleotides 136 to 138). Recent studies have shown that both proteins are synthesized and present in purified viruses (Chan et al., 1986). The second AUG conforms with Kozak's consensus sequence for a strong initiation site (Kozak, 1986) and is included within the N terminus hydrophobic region, but the
corresponding protein does not contain the putative signal peptide recognition site found between the two AUGs (Perlman & Halvorson, 1983). Thus, proteins synthesized using the second AUG have a molecular weight similar to the unglycosylated glycoprotein precursors from which the signal sequence has been cleaved. In fact, the molecular weight of SA11 VP7 initiated from the second AUG (35-3K) is similar to that of VP7 found in mutants of SA11 that are not glycosylated (Ericson et al., 1983; Estes et al., 1982). Glycosylation of VP7 is effectively inhibited in the presence of tunicamycin, indicating that carbohydrate side-chains are attached exclusively through N-linkage to asparagine residues (Kouvelos et al., 1984a, b). The glycosylated and non-glycosylated forms of VP7 of SA11, Wa and BDV 486 (calf rotavirus) strains differ in apparent molecular weight by 2500, 5000 and 6000 respectively (Kouvelos et al., 1984a, b). With an assumption of a contribution of 2500 mol. wt. per carbohydrate chain, it is likely that all the potential acceptor sites for N-linked carbohydrates (Asn-X-Ser) in these three strains are filled. The reason why strains like OSU and SA11 have one potential glycosylation site and HU/5 and UK have three, is unknown.

Preliminary characterization of native OSU VP7 glycoprotein in sucrose gradients indicates that it migrates as a high molecular weight aggregate (Gorziglia et al., 1985). The eight cysteine residues are conserved in all strains with the exception of one which is unique to SA11. Electrophoresis of purified OSU virions under reducing and non-reducing conditions suggests that VP7 has intramolecular but not intermolecular disulphide bonds, indicating that VP7 oligomers are not held together by this type of bonding (Gorziglia et al., 1985). Therefore, the cysteine residues may be involved in maintenance of the structural integrity of the glycoprotein. This arrangement could influence the exposure of potential epitopes. Sabara et al. (1985b) have shown that a 14K polypeptide fragment of the major bovine rotavirus glycoprotein is responsible for virus neutralization and cell attachment. This fragment was antigenic only in its unreduced conformation, indicating that disulphide bridges are necessary for maintaining antigenicity (Sabara et al., 1985a). This might explain why synthetic peptides that span the hydrophilic domains did not induce a significant neutralizing antibody response (Gunn et al., 1985).

The reported VP7 sequences from six rotavirus strains, which represent five serotypes, have considerable similarity in their long hydrophilic domains despite significant amino acid divergence. Antiserum prepared against synthetic peptides corresponding to the hydrophilic domain G provided low but reproducible neutralizing activity (Gunn et al., 1985). Amino acid residue 146 in hydrophilic domain E has a distinct amino acid substitution in each of the five serotypes, suggesting that it may also play a role in serotype specificity. Thus, more than one antigen-specific serotype site may exist. Nonetheless, the available sequence information concerning VP7 does not allow us to identify unequivocally serotype-specific sites on this glycoprotein.

Using monoclonal antibodies against the glycoprotein of SA11, Lazdins et al. (1985) selected mutants of SA11 that were resistant to neutralization by hyperimmune antiserum. Sequence analysis of VP7 from such mutants of different rotaviruses may ultimately lead to the identification of serotype-specific antigenic sites on VP7 that are involved in virus neutralization. Such studies are underway in our laboratory.

A serological survey from 75 Ohio swine herds indicated that 94% of the herds possessed antibody to the porcine OSU strain (Bohl et al., 1984). It has been reported (Bohl et al., 1984; Gaul et al., 1982) that cell culture-produced vaccines of OSU protected gnotobiotic pigs challenged with homologous but not heterologous virulent viruses. It is of interest that OSU strain is related antigenically in a two-way fashion to equine H-1 rotavirus (Hoshino et al., 1984). Thus, expression of OSU VP7 in a eukaryotic expression vector such as adenovirus (Davis et al., 1985), might provide a useful model to determine whether protection against rotavirus disease can be induced in pigs or horses by this glycoprotein.

We thank Sonia Gichner and Linda Jordan for editorial assistance in the preparation of this manuscript. This work is supported in part by grant SI-1311 from the Consejo Nacional de Investigaciones Cientificas y Tecnológicas (CONICIT), Caracas, Venezuela.
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(Received 17 February 1986)