MOLECULAR EPIDEMIOLOGY OF HUMAN ROTAVIRUSES - A STUDY IN GENETIC DIVERSITY

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Helsinki 2001

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II Maunula, L. and C.-H. von Bonsdorff. 1995. Rotavirus serotypes and electropherotypes in Finland from 1986 to 1990. Arch. Virol. 140(5):877-890.

III Maunula, L. and C.-H. von Bonsdorff. 1998. Short sequences define genetic lineages: Phylogenetic analysis of partial 4 and 9 sequences of group A rotaviruses. J. Gen. Virol. 79:321-332.

IV Maunula, L. and C.-H. von Bonsdorff. Frequent reassortments may explain the heterogeneity of rotavirus electropherotypes. (submitted).

In addition, some unpublished data are presented.

ABBREVIATIONS

aa	amino acid
bp	base pair
BSA	bovine serum albumin
CPE	cytopathic effect
DSP	dithiobis(succinimidyl propionate)
dsRNA	double-stranded RNA
EDTA	ethylene diamine tetraacetic acid
EIA	enzyme immunoassay
EM	electron microscopy
ER	endoplasmic reticulum
e-type	electropherotype
HI	haemagglutination assay
IFAT	indirect immunofluorescent antibody test
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
mw	molecular weight
nt	nucleotide
NT	neutralization test
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
p.i.	post infection
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RT-PCR	reverse transcription- polymerase chain reaction
$TCID_{50}$	tissue culture infectious dose
UTR	untranslated region
VLP	viruslike particle

SUMMARY

The molecular epidemiology of human rotaviruses infecting Finnish children was analysed with several molecular and genetic methods. Study material consisting of rotavirus-positive stools determined with electron microscopy (EM) was available for research. A seasonal pattern with a peak occurring in winter-springtime typical for rotavirus epidemics in temperate climates was seen. Over 3000 rotavirus-positive samples isolated during 1981-1997 were run in polyacrylamide gel electrophoresis (PAGE). In this material the electropherotypes (e-types) of 2835 group A and 20 group C rotaviruses were found. The remaining samples either contained more than 11 RNA segments (mixed e-types) or no RNA segments were visible.

Three of the 20 group C patients belonged to one family, and this family outbreak that occurred in 1990 was analysed in more detail. The disease had affected all five members of the family, although group C rotavirus was found only in the stools of the three children. In addition to the typical group C profile in PAGE from stool samples, group C rotavirus infection was verified from patient sera using a cell culture-adapted porcine group C rotavirus as a source of standard antigen. Different serological methods were employed for determination of antibody levels. Serum samples obtained from four family members were shown to contain IgG-class antibodies to group C rotavirus with the immunofluorescence antibody test (IFAT). Group C rotavirus-specific IgM-class antibodies were also present in the early serum samples of the three children. The presence of neutralizing antibodies against group C rotavirus in all the sera was also shown. These serological methods were found suitable for detecting group C rotavirus infections.

A detailed study of the group A rotavirus e-types of four epidemic seasons from 1986 to 1990 was undertaken. Over 600 isolates (83.9%) of 769 rotavirus-positive samples found in EM gave clear RNA patterns, which could be compared with those of the other e-types. The samples were grouped into 87 different e-types with a range of 16-34 e-types per season. In the epidemic season 1986/87 one e-type, serotype G1 rotavirus strain, was predominant over the other e-types in over 200 samples. Rotavirus strains of this e-type circulated throughout the entire season, but were no longer found in successive seasons. In season 1988/89 a new predominant e-type appeared, now a serotype G4 strain. This e-type was found during two epidemic seasons: 133 cases in season 1988/89 and 26 cases in the following season. This shift in serotypes from G1 to G4 was also observed in many other European countries at that time.

The G serotype distribution of the rotavirus strains was determined using VP7 protein-specific monoclonal antibodies (mAbs). By analysis of representatives of the same e-

types, each was found to be of the same G serotype. Assuming that this was true for all rotavirus strains, a distribution of serotypes G1-G4 of 61.2%, 2.0%, 0.5% and 29.8%, respectively, was obtained; 6.5% remained untypable.

Partial nucleotide sequencing of 109 serotype G1 and G4 e-types from the above four epidemic seasons was performed. The antigenically important regions of segments 4 and 9 coding for the VP4 and VP7 proteins were determined by sequencing, and the sequences obtained were compared using phylogenetic analysis. The VP4 sequences revealed the P genotypes of the rotavirus strains, which were all genotype P[8], with the exception of one strain that was of P[6] specificity. Phylogenetic analysis of the segment 9 sequences revealed that the G1P[8] rotavirus strains clustered into four lineages, and their segment 4 sequences clustered into two main lineages. The G4P[8] rotavirus strains did not form different segment 9 lineages, but their segment 4 sequences localized to the same two lineages as the G1 strains. The segment 4 and 9 lineage combinations of the e-types were determined. All combinations appeared to be possible, but certain combinations were preferred. The segment 9 lineages G1-1 and G1-2 were seen frequently in combination with segment 4 lineage P[8]-1, whereas lineage G1-3 preferred to combine with lineage P[8]-2.

To follow the molecular epidemiology of rotaviruses on a longer time-scale, the predominant e-types occurring over a period of 17 years was determined. During nine epidemic seasons one e-type predominated: in 1981, 1982, 1987 and 1997 it was serotype G1P[8] and in 1984, 1986, 1989, 1991 and 1993 it was serotype G4P[8]. In epidemic season 1983 one or several short serotype G2 e-types were more frequently found than long e-types. The nine G1 and G4 e-types were analysed in detail, and partial sequences from the variable parts of all 11 rotavirus RNA segments were determined. Phylogenetic analysis revealed that with the exception of segments 1, 3 and 10, the other segments could be assigned to 2-4 different clusters. The cluster combinations of the e-types were compared. It was found out that the predominant e-types did not share a common selection of RNA segments, but in contrast each different e-type had different genome segment combinations. These results suggest that segment reassortment is an important mechanism used by rotaviruses to create genetic variation. A high frequency of reassortments would also well explain the constant appearance and disappearance of different e-types that was so prominent a feature found throughout the long study period.

REVIEW OF THE LITERATURE

Classification of rotaviruses

Rotaviruses belong to the family Reoviridae together with the genera Phytoreo-, Fiji-, Cypo-, Aquareo-, Orbi-, Colti- and Orthoreovirus (Kapikian and Chanock, 1996). Viruses from the genera Orthoreo- and Rotavirus infect humans and animals. Common features occurring in the Reoviridae family are that 1) their genomes are formed from 10-12 dsRNA segments, 2) they are nonenveloped icosahedral viruses with two protein layers and a diameter of about 70 nm and 3) they replicate in the cytoplasm of cells.

Rotaviruses form seven antigenically different main groups that are named group A, group B, group C etc. (Table 1). Group A rotaviruses are the most common viruses infecting humans and also most animal species. Group B and C rotaviruses can also infect humans, but other groups have been found only in animal species; groups D, F and G rotaviruses are found only in birds. Rotaviruses belonging to different main groups appear identical in EM, although they diverge genetically. The three groups A, B and C have an amino acid homology ranging between 20 and 60%. Hyperimmune serum prepared against group A rotavirus does not react with group B or C rotaviruses. Human group B rotaviruses are found only in geographically limited areas; for many years they were found only in China (Hung et al., 1983; Hung et al., 1984), but recently they were also found in India (Krishnan et al., 1999). In China group B rotaviruses have caused large waterborne epidemics infecting thousands of people, mainly those 10-40 years of age (Hung, 1988). Group C rotaviruses circulate throughout the world but are much less common than group A rotaviruses. The main groups can be identified by their RNA patterns in PAGE, since their 11 RNA segments migrate differently. The RNA segments of group A rotaviruses form segment clusters in a pattern of 4-2-3-2 in which the first four longest RNA segments form cluster I (Figure 1). The RNA segments of group C rotaviruses migrate to a pattern of 4-3-2-2.

Some historical aspects

Human rotavirus was found in 1973 by Bishop and coworkers (1973). Virus particles with identical morphology were seen in biopsies of duodenal mucosae from nine children with acute nonbacterial gastroenteritis in thin sections by EM. The viruses were suggested to belong to the orbivirus group. A year later the same scientists reported that the viruses could also be found in negatively stained specimens of the faeces of ill patients, which made their detection much easier (Bishop et al., 1974). Viruses causing acute gastroenteritis in children and newborn calves were indistinguishable in size and shape (Flewett et al., 1974). The convalescent sera from

Table 1. *Classification scheme for human rotaviruses.*

Classification	Based on identification of
Groups A, B, C	Inner capsid VP6 antigens
Within group A	
Genogroup (Wa-like, DS1-like, AU1-like)	RNA-RNA hybridization
Subgroups (I, II)	Inner capsid antigens on VP6
G serotypes (G1, G2 etc.) (G = glycoprotein)	Outer capsid antigens on VP7
P serotypes (P1, P2 etc.) (P = proteinase)	Spike antigens on VP4
P genotypes (P[4], P[6] etc)	Sequence analysis of VP4
Electropherotypes (e-types)	Patterns of 11 genes after gel electrophoresis of genomic RNA

humans and calves agglutinated human and calf virus capsids in both directions in immunoelectron microscopy. It was suggested that these viruses should be named rotaviruses because of their wheellike appearance (rota = wheel in Latin).

Structure of rotaviruses

Soon after the discovery of human rotavirus, scientists were eager to determine the virus structure. Very detailed EM analyses were performed, but most of the results were later shown to be incorrect. Freeze-drying of the inner capsid revealed that the icosahedral pattern was compatible with a T=13 lattice (Roseto et al., 1979). This was later confirmed by the same technique and extended to T=13 laevo symmetry (Ludert et al., 1986). Metcalf (1982) had come to the same result concerning the symmetry of the reovirus outer shell. Despite the same triangulation number for rota- and reovirus, the detailed structure of these viruses was found to be very different (Metcalf et al., 1991).

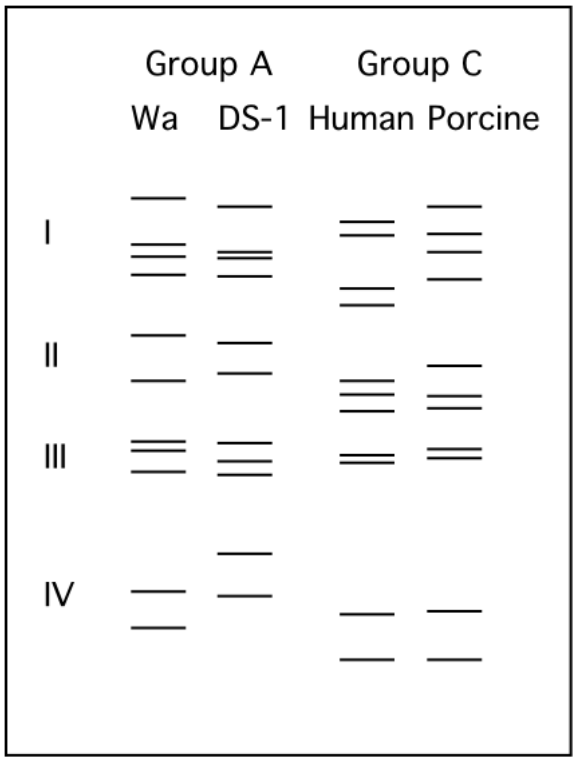


Figure 1. *Schematic representation of the RNA patterns of group A and group C rotaviruses in PAGE. Wa and DS-1 are human prototype strains for serotypes G1 and G2. They show a long and short electronpherotype (e-type), respectively.*

The three-dimensional structure of simian rotavirus SA-11 could be determined to a resolution of 40 Å by image processing of electron micrographs of virus particles embedded in vitreous ice (Figure 2; Prasad et al., 1988). The triangulation number of the icosahedral virion was confirmed as 13 in a left-handed configuration. The smooth outer surface of the virion was composed of 780 molecules of protein VP7 together with 60 spikes composed of protein VP4. The inner shell exhibiting a bristly surface was composed of 260 trimers of VP6. Both shells were spanned by 132 channels. The spike was shown to be a dimer of VP4 with a length of about 120 Å in cryoelectron micrographs of a complex between an intact virus and Fab fragments of a neutralizing mAb (Prasad et al., 1990). The three-dimensional structure of rhesus rotavirus (RRV) showed the same structural phenomena (Yeager et al., 1990).

The three-dimensional structure of the spike could be characterized in more detail after comparison of virions with spikes and reassortant virions without them (Shaw et al., 1993) or virions from which spikes were removed (Anthony et al., 1991; Yeager et al., 1994). The spikes were shown to be multidomained with a bilobed head and a

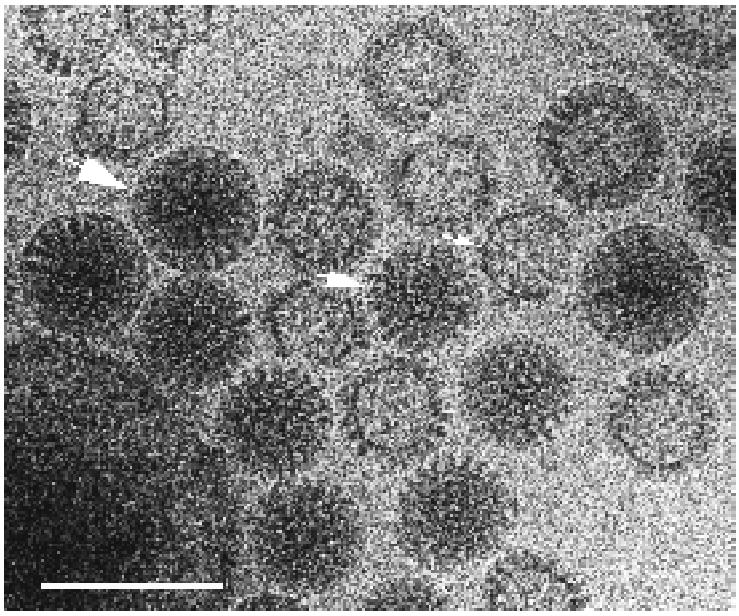


Figure 2. *Cryoelectron micrograph of group A rotavirus particles. Double-shelled (triple-layered) and single-shelled (double-layered) virus particles are indicated (big and medium arrow, respectively). Spikes are not resolved. Partly disrupted virus particles reveal the innermost VP2 shell (core or single-layered particle; small arrow). The dense particles have retained their RNA. Bar 100 nm. (From C.-H. von Bonsdorff, unpublished).*

radial length of about 200 Å. About half of the spike projected from the surface of the virus, while the penetrated part of it interacted with proteins VP7 and VP6. The ability to make VLPs (virus like particles) in baculovirus expression system has further aided cryo-EM analysis. Prasad et al. (1996) compared the empty VLPs and native virus particles and concluded that at least part of the genomic dsRNA (4500 bp) formed an ordered dodecahedral structure. The innermost layer of the triple-layered virion, the core layer, is formed of 60 dimers of VP2 and has a triangulation number of 1 (Lawton et al., 1997). Protein VP2 has been suggested to be arranged as dimers of 120 quasi-equivalent molecules.

EM and image processing have revealed that VP6 proteins form *p*6 plane group symmetry in the crystals (Hsu et al., 1997). The unit cell contains two VP6 trimers composed of three roughly circular subunits about 30 Å in diameter. Recombinant VP6 (rVP6) has been expressed, purified and crystallized (Petitpas et al., 1998). These crystals diffract to better than 2.4 Å and belong to the cubic space group $P4_132$ with a cell dimension *a* of 160 Å.

Aspects of morphogenesis

The first observations of human rotavirus morphogenesis were performed from duodenal biopsies (Holmes et al., 1975). The resemblance of rotavirus to reo- and orbiviruses was noted. Morphologically similar animal viruses such as Nebraska calf scours virus, EDIM virus (epizootic diarrhoea of infant mice) and the simian SA-11 virus had also been found earlier. Areas of viroplasm in cytoplasms of infected intestinal epithelial cells similar to those observed in reovirus-infected cells were encountered as a first sign of infection (Figure 3). Numerous virus particles were accumulated in the distended cisternae of the rough endoplasmic reticulum (RER). The virus particles were single-shelled (60 nm) or double-shelled (70-75 nm), and some also some appeared to be enveloped.

Saif et al. (1978) examined the morphogenesis of porcine rotavirus in porcine kidney cell cultures and in porcine epithelial cells by EM and confirmed the findings of Holmes et al (1975). They also suggested that virus particles acquired outer shells by budding through membranes of distended RER and observed the existence of core particles (electron-dense nucleoids with a diameter of 31-38 nm). Clark et al. (1980) found that L rotavirions (double-shelled light virions) were uncoated to D particles (single-shelled dense virions) and that newly formed D particles served as morphogenic precursors to L virions. The results were obtained by ^{3}H-labelling the L virions and determining the density of the virions by ultracentrifugation in CsCl gradients.

The tissue culture-adapted simian rotavirus SA-11 was the first virus that could be grown to high titres in the MA104 (foetal rhesus monkey kidney) cell line (Estes et al., 1979). Detailed EM analyses in this system suggested that assembly of rotaviruses occurred near the periphery of viroplasms after which virus particles entered the cisternae of the RER (Altenburg et al., 1980). The morphogenesis of human rotavirus Wa in the same cell line appeared identical to that of SA-11 (Suzuki et al., 1981). The existence of transiently membrane-enveloped intermediate particles has been established, and they were isolated from SA-11-infected MA104 cells (Poruchynsky and Atkinson, 1991). These intermediates comprised the proteins VP1, VP2, VP4, VP6, VP7, NSP4 and small amounts of NSP2 and NSP3.

Penetration, uncoating and release from the cell

The entry process of rotaviruses is still poorly known. It has been suggested that virus particles enter the cell through coated pits, coated vesicles and secondary lysosomes (receptor-mediated endocytosis; Ludert et al., 1987). Low pH did not cause the double-shelled virus to uncoat, which militates against the lysosomal route. Instead, low Ca^{2+}

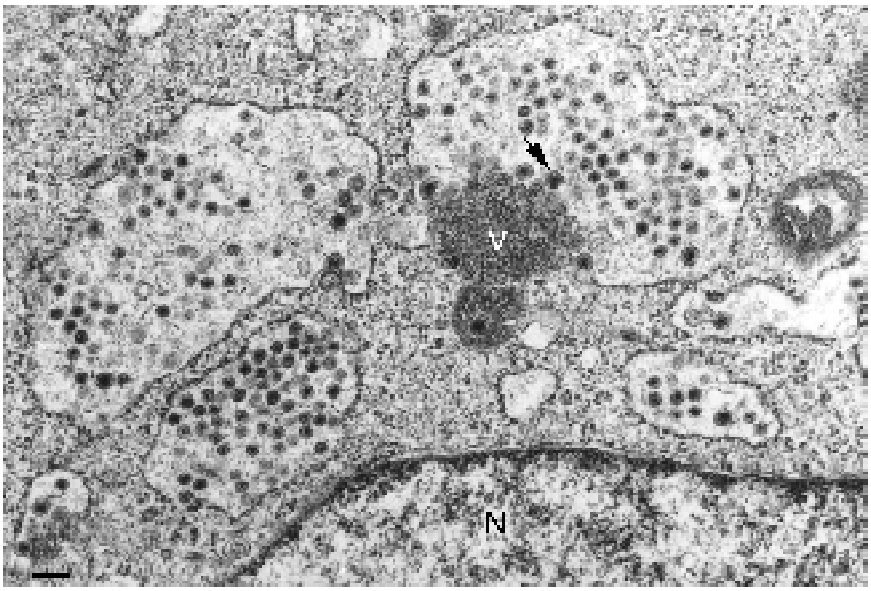


Figure 3. *Thin-section electron micrograph of a group A rotavirus-infected MA104 cell showing morphogenetic features of rotavirus replication. The dense granular area represents the viroplasm (V), in which the core particle is assembled. This buds through the ER membrane to form the transient enveloped particle (arrow). The dilatated ER-cisternae contain numerous mature virions that have shed off the envelope. N, nucleus, bar 200 nm. (From C.-H. von Bonsdorff, unpublished).*

concentration may cause virus uncoating, since it did not occur in increased intracellular Ca^{2+} concentration.

Results obtained in most cell culture studies have suggested that rotaviruses are retained in the endoplasmic reticulum (ER) lumen until cell lysis (Saif et al., 1978; Altenburg et al., 1980). In the intestinal human colon carcinoma cell line Caco-2, however, RRV was released from the cell within vesicles, since RRV release was blocked by carbonyl cyanide *m*-chlorophenylhydrazone which blocked vesicle transport (Jourdan et al., 1997). The virus did not go through the Golgi apparatus or lysosomes, since monensin or NH_4Cl did not affect its release, and RRV staining was never found in these organelles in confocal laser scanning microscopy.

General characteristics of the rotavirus genome

Since rotaviruses showed morphological resemblance to the orbi- and reoviruses as visualized by EM, it was easy to deduce that they would also have a similar segmented dsRNA genome. Calf diarrhoea virus was shown to have 11 dsRNA segments

(Newman et al., 1975; Rodger et al., 1975) as was human rotavirus (Schnagl and Holmes, 1976). The molecular weights of the RNA segments were determined with PAGE using the method of Loening (1969). It was found that detectable variations in the molecular weights of particular genome segments occurred in different isolates of human rotavirus (Schnagl and Holmes, 1976). RNA separation with PAGE soon became established as one of the most widely used methods in genetic and molecular epidemiologic analysis among rotaviruses. Differences between rotaviruses of different species as well as between rotaviruses of a single species could be detected by comparison of the genomes of simian, bovine and human rotaviruses and among bovine isolates (Rodger and Holmes, 1979). Hybridization experiments showed that on one hand animal and human rotavirus genome segments exhibited partial homology under conditions of low stringency (Schroeder et al., 1982), while on the other slight differences were evident even between rotavirus isolates of a single species under conditions of high stringency.

Molecular cloning techniques were adopted for rotaviruses (Both et al., 1982; McCrae and McCorquodale, 1982b; Gorziglia et al., 1983), which enabled the nucleotide sequence determination of the RNA segments and protein characterization after expression of the inserted plasmids in *E. coli*. The nucleotide sequence of segment 8 of the simian prototype rotavirus SA-11 was obtained by Both et al. (1982) and the entire SA-11 genomic sequence was completed in 1990 when segments 1 - 3 were sequenced (Mitchell and Both, 1990a; Table 2).

The genome of the SA-11 strain is AU-rich and has 18 555 bp of which only 6.3% are noncoding. The terminal structures of the RNA segments were analysed (Imai et al., 1983a; Spencer and Garcia, 1984). They have been shown to be base paired end-to-end and to contain the same terminal structures with a cap-structure in the 5'-terminus of the plus-strand:

5'(+) GGGC————————————GACC
3'(-) CCG————————————UGG

A conserved octanucleotide 5'-AUGUGACC-3' in the 3'-terminus of the plus-strand was found in human, bovine and porcine rotaviruses (McCrae and McCorquodale, 1983).

The secondary structure of rotaviral messenger RNAs (mRNAs) can play an important role in genome replication, assembly and translation. The secondary structures of SA-11 gene 8 and OSU gene 9 were predicted using the mfold program (Patton et al., 1993; Chen and Patton, 1998). The 5'- and 3'-ends appeared to form a panhandle structure by partial base-pairing with the exception that the 3'-terminal 12 residues

Table 2. *Group A rotavirus RNA segments and proteins (strain SA-11).*

RNA segment	Length (bp)	Protein	Length (aa)	MW	References
1	3302	VP1	1088	125 128	(Mitchell and Both, 1990a)
2	2690	VP2	881	102 698	(Mitchell and Both, 1990a)
3	2591	VP3	835	98 142	(Liu and Estes, 1989) (Mitchell and Both, 1990a)
4	2362	VP4	776	86 775	(Mitchell and Both, 1989)
5	1611	NSP1 (NS53)	495	58 484	(Mitchell and Both, 1990b)
6	1356	VP6	397	44 903	(Estes et al., 1984)
7	1104	NSP3 (NS34)	312	36 072	(Both et al., 1984)
8	1059	NSP2 (NS35)	317	36 629	(Both et al., 1982)
9	1062	VP7	326	37 198	(Both et al., 1983)
10	751	NSP4 (NS28)	175	20 309	(Both et al., 1983)
11	667	NSP5 (NS26)	198	21 772	(Mitchell and Both, 1988)

were nonbase-paired. The 5' UTR (untranslated region) formed a stem-loop motif. The initiation codon was not base-paired in the stem structure.

General characteristics of rotavirus polypeptides

Characterization of rotavirus polypeptides occupied rotavirus researchers during the late 1970s and early 1980s. Newman and coworkers (1975) could detect 5 proteins, two major and three minor proteins (mw 135-30 kDa) with PAGE, and more or less the same pattern was found by Cohen and coworkers (1979). The majority of researchers, however, felt that rotavirus particles contained eight structural proteins when polypeptides from purified virus particles were separated in PAGE (Rodger et al., 1975; Rodger et al., 1977; Kalica and Theodore, 1979; Matsuno and Mukoyama, 1979; Thouless, 1979). In infected cells three nonstructural proteins were often detected

(Matsuno and Mukoyama, 1979; Thouless, 1979). The 85-90-kDa polypeptide was considered to be an inner capsid protein until Espejo and coworkers (1981) showed that it was cleaved by trypsin into two smaller products and was an outer capsid protein. This polypeptide was known as VP3 for many years, since it often merged together with VP3 in PAGE until it was shown to be the fourth largest polypeptide VP4 (Dyall-Smith and Holmes, 1981).

Studies with radiolabelled virus grown in cell cultures and iodination of virus particles confirmed the earlier results and established VP2 (p91K) and VP6 (p45K) as the major proteins in the inner capsid, comprising about 20% and 80% of capsid proteins, respectively (Novo and Esparza, 1981). *In vitro* transcription and translation products were compared with polypeptides of double-shelled and single-shelled virions or polypeptides in infected cells (Mason et al., 1980; Arias et al., 1982). Ericson and coworkers (1982) detected five structural proteins (125, 94, 88, 41 and 38 kDa), three nonstructural proteins (53, 35 and 34 kDa) and two proteins (26 and 20 kDa) the assignment of which as to structural or nonstructural status remained tentative. The protein-coding assignment of the gene segments was revealed with the exception of RNA segment 3 (Mason et al., 1983). Already Rodger and coworkers (1977) had revealed the glycosylation of the major outer capsid protein (p34K) with the periodic acid-Schiff-test, and the result was confirmed by reaction with concanavalin A (Cohen et al., 1979) and by using tritium-labelled glycosamine (Mason et al., 1980). Later it was shown that two other proteins, NCVP5 and NCVP6 (currently referred as NSP4 and NSP5), were also glycosylated (Arias et al., 1982).

Proteins of group A rotaviruses

Structural proteins

VP1

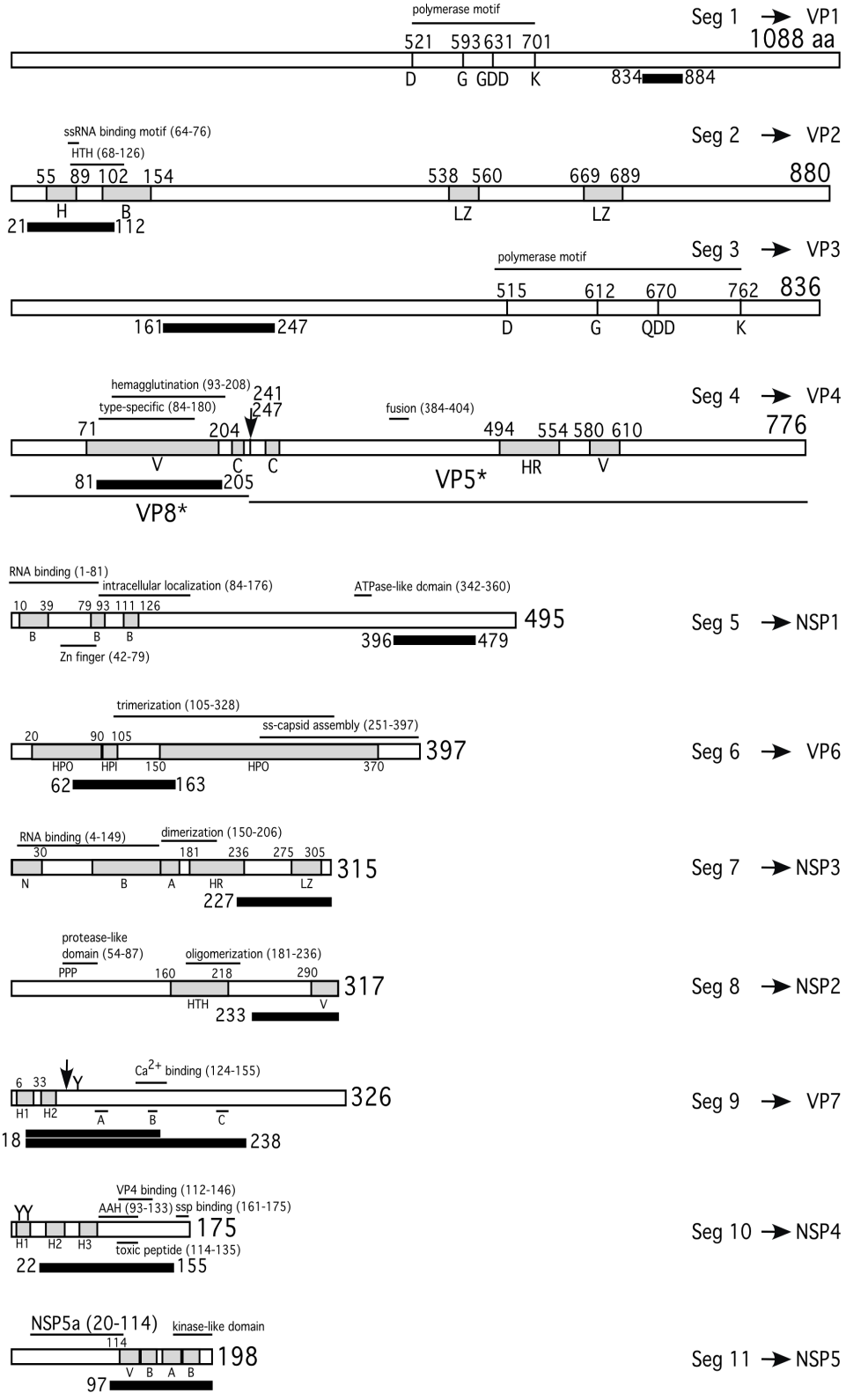
The largest polypeptide of rotavirus codes for the RNA polymerase (Figure 4, segment 1). The polymerase activity was shown to be associated with the purified virions of bovine and human rotaviruses (Cohen, 1977; Hruska et al., 1978). The polymerase was activated by a heat shock treatment (Cohen, 1977) or low concentration of a chelating agent such as EDTA, which solubilized the outer shell of the rotavirus and resulted in single-shelled viruses (Cohen et al., 1979). The replacement of ATP by its analogue β,γ-imido ATP resulted in complete inhibition of RNA synthesis, which suggested that ATP hydrolysable to ADP and P_i was required (Spencer and Arias, 1981). Treatment of the single-shelled virus particles with 0.5 M $CaCl_2$ removed the inner protein shell (VP6), leaving the viral core and resulted in disappearance of the

transcriptase activity (Sandino et al., 1986). The transcriptional activity could be restored by addition of VP6 in the absence of Ca^{2+}. The rotavirus polymerase was shown to synthesize ssRNA strands of only one polarity to the parental dsRNA template (Bernstein and Hruska, 1981; Patton, 1986), as did the reovirus polymerase. The transcripts formed did not have any polyadenylate sequences when examined with chromatography on an oligodeoxythymidylate column.

Cell-free systems supporting replication of the rotavirus resulting in synthesis of dsRNA were developed (Patton, 1986; Chen et al., 1994). The positive-sense ssRNA was used as a template in replication. While the single-shelled particles with the transcriptase activity were found at a density of 1.38 g / ml in CsCl gradients, particles with replicase activity were found in the pellet of the gradient (Helmberger-Jones and Patton, 1986). These particles contained a core of VP1 and VP2, but less VP6 protein per particle than those with transcriptase activity. The presence of divalent metal ions, e.g. Mg^{2+}, was found to be obligatory for polymerase activity both in transcription and replication (Cohen, 1977; Chen et al., 1994).

The nucleotide sequence of gene segment 1 revealed that it shared consensus sequences with several well-characterized RNA-dependent RNA polymerases (Cohen et al., 1989; Fukuhara et al., 1989; Mitchell and Both, 1990a). Fukuhara and coworkers (1989) also found consensus sequences of GTP-binding proteins in the VP1 gene, but no guanylyltransferase activity has been associated with VP1 protein. A photoreactive nucleotide analogue was used to identify the viral polymerase (Valenzuela et al., 1991). Using photoreactive, radiolabelled GTP, incorporation of radioactivity into VP1 protein could be observed upon UV-treatment of single-shelled virus particles and this was associated with overall decrease in rotavirus mRNA synthesis. These results indirectly

Figure 4. *Protein products of 11 RNA segments of group A rotavirus. The numbers indicate amino acid positions. Several features of the proteins are indicated; A, acidic region, AAH, amphipathic alfa-helix, B, basic region, C, constant region, H, charged helix, HPI, hydrophilic region, H1-H3, HPO, hydrophobic region, HR, heptad repeat, HTH, helix turn helix, LZ, leucine zipper, N, noncharged amino acids, PPP three prolines, V, variable region. The antigenic regions A, B and C of VP7 are indicated by bar under the schematic. The areas corresponding to the RNA sequences that were determined in this study are shown by black boxes. Both the longer and shorter region of VP7 that were determined in study III and IV, respectively, are shown. All except the three longest polypeptides are shown in the same scale.*


polymerase motif
Seg 1 → VP1
521 593 631 701
1088 aa
D G GDD K
834 884
ssRNA binding motif (64-76)
HTH (68-126)
Seg 2 → VP2
55 89 102 154
538 560
669 689
880
H B
LZ LZ
21 112
polymerase motif
Seg 3 → VP3
515 612 670 762 836
161 247
D G QDD K
hemagglutination (93-208)
241
type-specific (84-180)
247
fusion (384-404)
Seg 4 → VP4
71 204
494 554 580 610
776
V C C
HR V
81 205
VP5*
VP8*
RNA binding (1-81)
intracellular localization (84-176)
ATPase-like domain (342-360)
10 39 79 93 111 126
495
B B B
396 479
Zn finger (42-79)
Seg 5 → NSP1
trimerization (105-328)
ss-capsid assembly (251-397)
20 90 105
397
HPO HPI 150 HPO 370
62 163
Seg 6 → VP6
RNA binding (4-149)
dimerization (150-206)
30 181 236 275 305
315
N B A HR LZ
227
Seg 7 → NSP3
protease-like
domain (54-87)
oligomerization (181-236)
PPP 160 218 290
317
HTH V
233
Seg 8 → NSP2
Ca2+ binding (124-155)
6 33
326
H1 H2 A B C
18
238
Seg 9 → VP7
VP4 binding (112-146)
AAH (93-133) ssp binding (161-175)
175
H1 H2 H3
toxic peptide (114-135)
22 155
Seg 10 → NSP4
NSP5a (20-114)
kinase-like domain
114
198
V B A B
97
Seg 11 → NSP5

confirmed that VP1 was the rotavirus RNA-dependent RNA polymerase. In gel mobility shift assay, VP1 was the only viral component found in a protein-RNA complex when replication of the 3'-end mRNA of segment 8 was examined (Patton, 1996). VP1 also recognized the 3'-end of segment 8 in the absence of other core proteins.

The replicase activity of VP1, however, required the presence of the core stucture of VP2 (Patton, 1996). Studies on VLPs formed by rotavirus proteins expressed separately in the baculovirus system revealed that VP1 was needed for replicase activity of the VLPs, and the minimal replicase particle was constituted by VP1 and VP2 (Patton et al., 1997; Zeng et al., 1998). The VP1 protein may be bound to the N-terminus of VP2 (aa 1-25) at least transiently, since the DVP2-VLPs formed from truncated VP2 lacking the N-terminus did not encapsidate VP1 and VP3 but resulted in empty VP2-VLPs although the full-length VP2 formed VP1-VP2-VP3-VLPs (Zeng et al., 1998). The essential cis-acting signal for replication was determined to be the terminal 26 nt of the 3'-end of the template RNA (Chen et al., 1994). Virtually the same region, 3'-terminal 19 nt, was shown to restore amplification and expression of complementary DNA (cDNA) constructs in which the chloramphenicol acetyltransferase reporter gene was flanked by rotavirus 5'- and 3'-terminal noncoding regions in rotavirus-infected cells (Gorziglia and Collins, 1992). The minimal promoter of the minus-strand RNA synthesis was contained in the 7 terminal nucleotides of the 3'-end in segment 9 transcripts of porcine rotavirus OSU (Wentz et al., 1996), and these were conserved in all gene segments. The synthesis of dsRNA was only slightly decreased by the absence of the wild-type 5' stem-loop (Chen and Patton, 1998). The nonstructural NSP2 protein that is involved in viral replication also interacted with VP1 based on DSP-crosslinking of infected cell lysates and immunoprecipitation (Kattoura et al., 1994).

VP2

VP2 is the gene product of segment 2, and VP2 proteins form the rotavirus core particle within which are enclosed the proteins VP1 and VP3 and the 11 genome segments. VP2 as well as VP6 were shown to be myristylated in an amide bond upon labelling with myristic acid during replication (Clark and Desselberger, 1988). The functional significance of the myristylation is not known, but VP2 of polyoma virus (Streuli and Griffin, 1987) and VP4 of picornavirus (Chow et al., 1987) are also myristylated and they function as scaffolding proteins. The nucleotide sequence of VP2 revealed a putative leucine zipper (Kumar et al., 1989), and the hydrophilic N-terminal region of VP2 was predicted to extend over 10 turns of an α-helix (Ernst and Duhl, 1989). Oppositely charged residues were arranged at positions 1 and 4 of the helix, which may allow dimerization of VP2 via alignment of parallel helices (Mitchell and Both, 1990a).

The RNA overlay-protein blot assay showed that protein VP2 was able to bind RNA (Boyle and Holmes, 1986). The binding was not sequence-specific, but ssRNA was preferred over dsRNA. The RNA-binding capacity was also found in empty corelike particles formed by the baculovirus-expressed rVP2 (Labbé et al., 1991). VP2 has also been expressed by vaccinia (Gonzalez and Affranchino, 1995) and Semliki Forest virus (Nilsson et al., 1998) expression systems. The nucleic acid-binding domain of VP2 was shown to localize between amino acids 1-132 by blotting experiments with truncated rVP2 (Labbe et al., 1994). The N-terminus of VP2 (aa 1-25) was also shown to be necessary for encapsidation of VP1 and VP3 (Zeng et al., 1998; see section on VP). Localization of the aminoterminal half of VP2 within the core was studied with cryo-EM (Lawton et al., 1997). The results suggested that the aminotermini of the VP2 dimers localize in the vicinity of the icosahedral vertices when the three-dimensional structures of the intact and amino-terminal–deleted VP2 particles were compared.

In rVP2 particles (Labbé et al., 1991; Gonzalez and Affranchino, 1995) as well as in empty rotavirus capsids purified from a rotavirus-infected cell culture (Brussow et al., 1990), cleavage products of VP2 (90 kDa and 85 kDa) were found. These cleavage products may be identical to the cleavage products (88 kDa and 84 kDa) that were observed after exposure of single-shelled rotavirus particles to proteolytic enzymes *in vitro* (Estes et al., 1981). The absence of RNA interacting with VP2 was suggested to make the protein susceptible to proteases (Brussow et al., 1990). Further characterization of the rVP2 particles revealed the presence of three major VP2-related proteins of 94 kDa, 85 kDa and 77 kDa (Zeng et al., 1994).

The concentration of rVP2 particles converted them to unusual forms, including elongated bristly structures, helix-like structures and sheetlike helix structures. The structures could be converted to particles by dilution. Native core particles were shown to behave similarly with the exception that the phenomenon was irreversible. The biologic relevance of the structural conversions is not known, but it has been suggested that it may regulate capsid formation during morphogenesis (Zeng et al., 1994).

VP2 was shown to be necessary for viral replicase activity, since the level of this activity was greatly decreased in subviral particles of the tsF (temperature sensitive) mutant mapping to gene segment 2 (Mansell and Patton, 1990). The importance of VP2 in replicase and transcriptase particles has been established (see section on VP1). It may play a role as a scaffolding protein, since it has no known enzymatic activity.

A nonneutralizing mAb YO-60, which agglutinated only single-shelled particles, was found to be directed to VP2 (Taniguchi et al., 1986). Analysis of rotavirus strains with

this mAb suggested the possible presence of a subgroup (sg) antigen on VP2. The sg I antigen was detected both in human rotaviruses and rotaviruses of all animal species analysed, but sg II antigen was detected only in human and porcine rotaviruses (Svensson et al., 1990). A strong immune response against VP2 as well as VP6 is common, since IgG and IgA responses against them have nearly always been found in sera from individuals infected with rotavirus when detected with immunoblotting (Ushijima et al., 1989; Richardson and Bishop, 1990) or by RIPA (Johansen et al., 1994; Colomina et al., 1998).

VP3

Gene segment 3 of rotavirus SA-11 codes for VP3 which is a guanylyltransferase. Identification of VP3 was delayed compared with the other proteins, because the gene segment often comigrated with segment 4 as did the VP3 protein with VP4 in gel electrophoresis and VP3 was translated in lower amounts than VP4. The present VP4 was termed VP3 until 1988, when Liu and coworkers (1988) showed that the SA-11 genome segment 3 coded for a protein with an apparent mw of 88 kDa by using conditions of electrophoresis that improved resolution of high-molecular-weight proteins. VP3 was shown to be located in viral core particles as a structural protein (Liu et al., 1988).

The VP3 protein was suggested to play a role in RNA replication, since the ts mutant B mapping to segment 3 had an RNA-negative phenotype (Gombold et al., 1985; Gombold and Ramig, 1987), and it was found in early replication intermediates (Gallegos and Patton, 1989). VP3 is not, however, required for replication activity, since VLPs formed by VP1 and VP2 supported replication (Zeng et al., 1996). Although the nucleotide sequence revealed similarity of VP3 both with RNA polymerases (Liu and Estes, 1989; Mitchell and Both, 1990a) and GTP-binding proteins (Fukuhara et al., 1989), it was shown to possess GTP-binding but not polymerase activity (Fukuhara et al., 1989). VP3 was shown to form a stable complex with nucleotide analogues that inhibited *in vitro* transcription (Pizarro et al., 1991b). More detailed characterization of the rotavirus guanylyltransferase VP3 indicated that the presence of Mg^{2+} or Mn^{2+} was necessary for activity and in the presence of Mg^{2+} the enzyme accepted only GTP or dGTP, but that in the presence of Mn^{2+} several other nucleotides were accepted (Pizarro et al., 1991a). The recombinant rVP3 has also been shown to bind GTP covalently and reversibly both alone and in VLP particles lacking nucleic acids (Liu et al., 1992).

VP3 showed affinity only for ssRNA, not for dsRNA, in gel shift analysis performed with disrupted virion-derived cores and virus-specific RNA probes (Patton and Chen,

1999). The ssRNA-binding activity was found to be sequence-independent. VP3 exhibited a slight preferential affinity for uncapped over capped RNA, and it could cap RNAs initiating with a G or an A residue (Patton and Chen, 1999). VP3 was suggested to be a multifunctional enzyme with both guanylyltransferase and methyltransferase activity (Chen et al., 1999). The studies were performed in an open core-capping system that appeared to lack RNA 5'-triphosphatase and nucleoside triphosphatase activity, since the majority of the caps contained the tetraphosphate linkage GppppG instead of the triphospate linkage GpppG found on native rotaviral mRNA.

VP4

VP4 is a multifunctional spike protein with haemagglutinating, neutralizing and fusion activities and antigenic properties. The 88-kDa structural protein is cleaved into two smaller polypeptides, 60 kDa and 28 kDa, by trypsin (Clark et al., 1981; Espejo et al., 1981; Estes et al., 1981). These cleavage products are called VP5* and VP8*. This enhances the infectivity and plaque formation of rotaviruses, a property that was shown to be associated with the fourth RNA segment. The cleavage site in VP4 was determined to be located in the region of aa 241-247 (Lopez et al., 1985; Arias et al., 1987). The mechanism governing how infectivity was increased by the cleavage of VP4 was studied, and it was shown that virus attachment to cells was independent of trypsin treatment (Clark et al., 1981; Fukuhara et al., 1988). Clark and coworkers (1981) suggested that noninfectious virus was converted to an infectious form by allowing it to uncoat in the infected cell. Fukuhara et al. (1988) concluded that trypsin treatment was needed for the selective internalization of the inner capsid components, since mainly only inner capsids were found in untreated cells after internalization. In the presence of trypsin the replication of rotaviruses was found to increase in cell cultures (Babiuk et al., 1977; Theil et al., 1977), and with this technique the first human rotavirus, Wa strain, could be grown at high titres in AGMK (African green monkey kidney) cells (Wyatt et al., 1980).

VP4 has also been suggested to play a role in virulence. The development of diarrhoea in newborn mice inoculated orally with bovine NCDV or simian SA-11 strains or reassortant viruses was inspected (Offit et al., 1986a). It was found that the dose of SA-11 and NCDV rotavirus required to induce gastroenteritis was determined by gene segment 4. The fact that rotavirus strains M37, 1076, McN13 and ST3 isolated from neonates with asymptomatic infection all possessed highly related VP4 genes and were distinct from rotavirus strains Wa, DS-1, P and VA70 that cause gastroenteritis (Flores et al., 1986; Gorziglia et al., 1986b; Gorziglia et al., 1988a) support this hypothesis.

Gene segment 5, however, have also been reported to be associated with virulence (Broome et al., 1993). Recently, both virulence and host range restriction have been suggested to be multigenic phenotypes in rotaviruses (Broome et al., 1993).

Trypsin cleavage of VP4. The first attempt to locate the trypsin cleavage site of VP4 was performed by amino acid sequencing of the amino terminus of the cleavage product VP5* (Lopez et al., 1985). It was discovered that VP5* contained two polypeptides that differed only by the presence of six extra amino acids. A conserved trypsin cleavage site, located at aa 247, was determined in RRV VP4 (Mackow et al., 1988b). A total of three sites susceptible to trypsin (Arg-241, Arg-231 and Arg-247) were found, but only site arg-247 was associated with enhancement of infectivity (Arias et al., 1996). The VP4 end products were a homogeneous VP8* polypeptide comprising aa 1-231 and a heterogeneous VP5*, which is formed by two polypeptide species (at a ratio of 1:5) as a result of cleavage at either Arg-241 or Arg-247. Trypsin activation was found to be necessary for rotavirus to mediate cell-cell fusion (Falconer et al., 1995). Elimination of the three Arg residues by site-directed mutagenesis prevented syncytium formation (Gilbert and Greenberg, 1998). Only Arg-247 appeared to be required for activation of VP4 functions and cell-cell fusion.

Results from analyses of murine EDIM strain in mice indicate that VP4 is cleaved upon release from the intestinal cell and that virus shed into the environment does not have an intact VP4 (Ludert et al., 1996). When RRV was grown in the presence of trypsin inhibitors and the cleavage of VP4 was compared in orally infected adult and suckling mice, differences in cleavage patterns were detected (Bass et al., 1992). They suggest that the susceptibility of the infants for rotavirus infection might partly be due to differences in the content of their gastric acid and pepsin secretion as compared with adults.

Haemagglutination. The ability to haemagglutinate was associated with a segment 4 product and VP4 (Greenberg et al., 1983b; Kalica et al., 1983). Animal rotaviruses such as simian SA-11 and bovine NCDV could haemagglutinate human O-erythrocytes, but human rotaviruses could not. Human rotaviruses (Wa, KUN, MO) showed haemagglutination only with fixed 1-day-old chicken erythrocytes, which was suggested to be independent of sialic acids (Kitaoka et al., 1986; Fukudome et al., 1989). The haemagglutination of fresh erythrocytes appeared to be sialic acid-dependent, since it was inhibited by neuraminidase treatment (Bastardo and Holmes, 1980; Fukudome et al., 1989). The human erythrocyte protein, glycophorin, inhibited haemagglutination by RRV or the expressed VP4 protein and was suggested to be the rotavirus erythrocyte receptor (Mackow et al., 1989).

MAbs against VP4 that inhibited haemagglutination but lacked neutralizing activity could be generated (Burns et al., 1988). Baculovirus rVP4s from simian RRV (Mackow et al., 1989) and porcine OSU strains (Nishikawa et al., 1989a) were generated, and proteins with the same characteristics as the native proteins were expressed. Haemagglutination assays with chimeric polypeptides MS2-VP8* in *E. coli* (Lizano et al., 1991) and rVP8* in baculovirus (Fiore et al., 1991) showed that VP8* alone showed haemagglutinating activity, but VP5* did not. Studies with chimeras between the VP4 genes of haemagglutinating (YM) and nonhaemagglutinating (KU) rotavirus strains indicated that the region aa 93-208 of YM VP4 was sufficient to determine the haemagglutination activity (Fuentes-Panana et al., 1995). When amino acid substitutions for tyrosines in this region arose by *in situ* mutagenesis, alanine substitutions for tyrosines 155 and 188 and for serine 190 abolished the haemagglutination acitivity but did not affect the overall conformation of the protein, as judged by mAbs against VP8* (Isa et al., 1997).

Fusion. The VP5* neutralization region reported by Mackow was similar to the putative fusion sequences of Sindbis and Semliki Forest viruses (Mackow et al., 1988b). The fusion activity was examined with liposomes containing encapsulated self-quenching concentrations of the fluorophore carboxyfluorescein (CF; Nandi et al., 1992), isolated membrane vesicles from apical membrane of pig enterocytes containing CF (Ruiz et al., 1994) and cell-cell fusion detection in cell cultures supplemented with cholesterol (Falconer et al., 1995). In all systems the presence of outer membrane proteins VP4 and VP7 in the virions was necessary as was cleavage of VP4 by trypsin for fusion to occur. The fusion activity was also present in VLPs formed by rVP2/4/6/7 after trypsin treatment in cholesterol-supplemented cell culture systems (Gilbert and Greenberg, 1997). Trypsinization of VP4 was required, since elimination of all three trypsin-susceptible arginine residues or Arg-247 of VP4 by specific site-directed mutagenesis prevented syncytium formation (Gilbert and Greenberg, 1998). VP5* was suggested to contain the fusion region, since mAbs against it inhibited the reaction in liposomes (Ruiz et al., 1994). Expressed rVP5* could alone permeabilize large unilamellar vesicles preloaded with CF, but VP4 or VP8* could not (Denisova et al., 1999). Studies with truncated VP5* indicated that the fusion region included aa 265-474 (Denisova et al., 1999).

Neutralization. In addition to VP7, VP4 was also found to have neutralizing activity and production of mAbs against VP4 of different animal and human rotavirus strains was produced. Cross-reactive neutralizing mAbs for human rotavirus strains S2, KU and YO were produced (Taniguchi et al., 1985), and distinct epitopes were found (Taniguchi et al., 1987a). The cross-reactive neutralization epitopes were localized in

VP5* (aa 305, 433 and 439) by determining the mutated amino acids in variants that escaped neutralization (Taniguchi et al., 1988b). MAbs against VP8* of human rotavirus KUN neutralized the infectivity and inhibited haemagglutination (Kitaoka et al., 1986). MAbs against the animal rotaviruses simian SA-11, RRV and porcine OSU or against rVP4 or their cleavage products were also produced (Greenberg et al., 1983b; Offit et al., 1986b; Burns et al., 1988; Shaw et al., 1988; Nishikawa et al., 1989a). Mackow and coworkers (1988b) found five regions in VP8* associated with mainly strain-specific neutralization (aa 87-188) and one region in VP5* that neutralized a broad range of serotypes in simian RRV (aa 388-393). The amino acids 247-474 were shown to be required for binding three serotype-specific mAbs (2G4, M7, M2; VP5*), when truncated RRV VP4 polypeptides of different lengths were tested (Mackow et al., 1990). A broadly cross-reactive neutralizing antibody response was obtained against expressed rVP8* protein (RRV) in mice (Fiore et al., 1991).

It became evident that VP4 serotypes existed and that they segregated independently of VP7 serotypes (Gorziglia et al., 1990). Rotaviruses exhibiting a VP4 amino acid identity of 89% or greater belonged to the same VP4 serotype as determined by neutralization. When antibodies against rVP8* and rVP5* polypeptides of different strains were analysed, only the antibodies against rVP8* showed serotype-specific neutralization activity (Larralde et al., 1991).

VP6

VP6 forms the inner capsid of rotaviruses. Initially, rotaviruses were found to share one or more common antigens by complement fixation, immunofluorescence and immuno-EM (Kapikian et al., 1976; Woode et al., 1976). Later, more specific antigens were detected by neutralization, haemagglutination and EIA (Thouless et al., 1977; Yolken et al., 1978). Analysis of reassortants between bovine UK and human Wa rotaviruses with different techniques revealed that the ninth segment (VP7) segregated with the neutralization specificity and the sixth segment (VP6) segregated with the subgroup antigenic specificity detected in the immune adherence haemagglutination assay (Kalica et al., 1981; Kapikian et al., 1981).

MAbs directed to VP6 were produced and monoclones with sgI and sgII specificities were selected (Greenberg et al., 1983a). Both sgI and II specificities were detected among human rotaviruses, although sgII was more common. Most animal rotaviruses carried sgI specificity with the exception of porcine strain Gottfried (sgII). Murine EDIM rotavirus did not react with either of the mAbs (non-sgI/II; Greenberg et al., 1983a), and some equine rotaviruses (FI-14) were found to bear both sgI and sgII specificities (Hoshino et al., 1987). When human rotavirus Wa (sgII) and animal

rotaviruses SA-11 and RF (sgI) were sequenced, over 90% of their amino acids were found to be conserved (Both et al., 1984b; Cohen et al., 1984; Estes et al., 1984). Most of the amino acid differences between the strains SA-11 and Wa were located in three regions: aa 39-62, aa 80-122 and aa 281-315 (Both et al., 1984b).

VP6 was found to be trimeric, since VP6 proteins removed from virus particles with $CaCl_2$ had a sedimentation coefficient compatible with an oligomeric structure in sucrose density gradient centrifugation (Gorziglia et al., 1985). Native VP6 present in the infected cells was also in oligomeric form. The rVP6 protein expressed in baculovirus maintained native antigenic determinants and spontaneously assembled into morphologic subunits (Estes et al., 1987). MAbs reacting with both monomeric and trimeric VP6 or only with the trimeric form were produced (Gerna et al., 1989). VP6 proteins alone or together with VP2 could spontaneously form single-shelled capsid-like particles (Estes et al., 1987; Gonzalez and Affranchino, 1995). Rotaviruslike particles were also produced after coexpression of VP6/7 (Sabara et al., 1991), VP2/4/6, VP2/6/7 or VP2/4/6/7 (Crawford et al., 1994).

The antigenic sites of VP6 have been intensively studied. The subgroup epitopes have been suggested to be mainly conformational, since most of the mAbs reacted only with trimeric VP6. The results from different epitope studies have been rather confusing. Five epitopes were found with VP6-specific mAbs using a competitive binding assay (Pothier et al., 1987). Four non-overlapping antigenic domains were detected, and three of the epitopes were common to all rotavirus strains. Kohli and coworkers (1992) studied immunoreactivity of synthetic peptides that contained bovine rotavirus VP6 sequences, and their results indicated that the sequence of aa 48-75 would be present in one of the immunodominant sites of VP6. After comparison of amino acid sequences of rotavirus strains with different subgroup specificities, five regions contributing to subgroup epitopes were defined (Gorziglia et al., 1988b). Regions A (aa 45 and 56) and C (aa 114 and 120) contributed to sg I and regions B (aa 83, 86, 89 and 92), D (aa 312 or 314, 317 or 319) and E (aa 341 or 343, 350 or 352) contributed to sg II (Gorziglia et al., 1988b). MAbs produced to porcine OSU VP6 (sgI) fell into six patterns of reactivity, three of which recognized a single antigenic site, which included at least three overlapping epitopes (Liprandi et al., 1990).

Even a single amino acid mutation (aa 172 or 305) has been shown to be sufficient to change an subgroup specificity of rotavirus VP6 protein (Lopez et al., 1994). Interactions of mAbs to VP6 with purified viral particles were studied using the BIAcore methodology (Tosser et al., 1994). As expected, several mAbs reacted with single-shelled particles. MAbs that did not react with single-shelled particles were also

detected. Interestingly, one mAb reacted with both single-shelled and double-shelled particles inducing a significant decapsidation to the latter particle type. Tang and coworkers (1997) searched regions involved in subgroup specificity by site-specific mutagenesis experiments and identified amino acid positions 305, 315 and a region of aa 296-299. They also confirmed the finding of Lopez that a single amino acid mutation could change the subgroup specificity (aa 305 and 315).

The domain for trimerization was defined to reside near the centre of VP6 located between aa 105-328, when truncated VP6 proteins were examined by electrophoresis assay for the ability to trimerize *in vitro* (Clapp and Patton, 1991). Recently, region aa 246-314 was found to be critical for oligomerization by using VLPs VP2/DVP6 with in-frame deletions (expressed in vaccinia; Affranchino and Gonzalez, 1997). A mutation in aa 309 has been shown to affect the stability of the rearranged lamb rotavirus VP6 trimers (Shen et al., 1994). The region aa 251-397 of VP6 was required for the formation of single-shelled capsids (Clapp and Patton, 1991).

Although VP6 is not required for replicase activity (Mansell and Patton, 1990), it is necessary for trancriptase activity. Transcriptase activity could be reconstituted by using core particles and rVP6 protein (Kohli et al., 1993). How VP6 functions in transcription is not known. MAbs to VP6 could be produced that inhibited transcription *in vitro* (Ginn et al., 1992; Kohli et al., 1994). One of the mAbs was shown to recognize aa 56-58 of VP6 (Sandino et al., 1994).

During rotavirus infection the strongest immune response is usually formed to VP6 protein both in humans (Svensson et al., 1987a, 1987b; Ushijima et al., 1989; Richardson and Bishop, 1990; Colomina et al., 1998), and animals (Ishida et al., 1997). Antibody responses to VP6 were rapid, increased in intensity during 20-40 days after the onset of symptoms and persisted for more than 4 months when detected, using immunoblotting in sera obtained from children with acute rotavirus diarrhoea (Richardson and Bishop, 1990). VP6 was also the most immunogenic protein both in sera and in stools during rotavirus infection in mice (Ishida et al., 1996).

The heterotypic cell-mediated clearance of rotavirus in SCID mice was shown to be mediated by proteins VP1, VP4, VP6 and VP7, which induced $CD8^+$ T lymphocytes (Dharakul et al., 1991). Cytotoxic T-cell epitopes (Franco et al., 1994) and T-helper cell epitopes (Banos et al., 1997) have been identified on the VP6 proteins. A VP6 fragment (aa 283-307) containing a $CD4^+$ cell epitope could stimulate nearly complete protection in BALB/c mice (Choi et al., 2000). Evidence has been obtained that VP6 can prime for an enhanced neutralizing antibody response, and thus T-helper cells specific for

VP6 could provide cognate help to B cells specific for neutralizing epitopes on VP7 and / or VP4 (Esquivel et al., 2000).

A backpack tumour transplantation model in mice has been used to determine the mechanism by which secretory IgA provides protection *in vivo* (Burns et al., 1996). The nonneutralizing IgA antibodies to VP6 were not able to inhibit primary rotavirus replication when directly administered on the luminal site of the intestine, but prevented primary infection in mice carrying backpack tumours (multiply cloned IgA hybridoma cell lines reactive with VP6). The observations supported the hypothesis that the antirotaviral effect occurred during IgA transcytosis in cells rather than as an extracellular event in the gut lumen.

VP7

VP7 forms the outer protein layer of the rotavirus. The protein was found to be glycosylated (Matsuno and Mukoyama, 1979; Mason et al., 1980). VP7 glycoprotein was shown to be of high mannose type, since it could be labelled with radiolabelled glucosamine or mannose (Ericson et al., 1982), cleaved by endo-b-N-acetylglucosaminidase H (Arias et al., 1982) and viruses could be aggregated by concanavalin A (Svensson, 1984). Processing of the molecules was shown to proceed in a time-dependent manner, so that $Man_8GlcNac_2$ and $Man_6GlcNac_2$ were the predominant intracellular species and $Man_5GlcNac_2$ was found on mature virus (Kabcenell and Atkinson, 1985). Previously, Kouvelos and coworkers (1984) had detected VP7 with N-linked Man_7-residues on mature virus. A single glycosylation site was found to localize at aa 69-71 of simian SA-11 rotavirus VP7 (Both et al., 1983a).

In a cell-free system a 37-kDa VP7 protein, which contained a cleavable signal sequence, was synthesized and after glycosylation processing *in vitro* a final 38-kDa VP7 product was detected (Ericson et al., 1983). Despite the presence of the signal sequence, VP7 was found to be retained in the ER (Petrie et al., 1982), where it was present as an integral membrane protein (Kabcenell and Atkinson, 1985). The amino acid sequence of VP7 revealed two hybrophobic regions, H1 (aa 6-23) and H2 (aa 32-48), in the NH_2-terminus, and one in-frame initiation codon preceded each of the regions (Both et al., 1983a). Only the latter initiation codon (Met-30) was used, and the following signal peptide including H2 was cleaved between Ala50 and Gln51 (Stirzaker et al., 1987). VP7 deletion mutants affecting the H2 region were not retained in the ER, but were transported and secreted, whether they were glycosylated or not (Poruchynsky et al., 1985). On the other hand, a chimera in which the VP7 NH_2-terminus (aa 14-111) was fused to the amylase gene, was retained in the ER, although normally amylase was

secreted (Poruchynsky and Atkinson, 1988). If the H2 signal peptide was replaced by one from influenza haemagglutinin, VP7 was rapidly secreted (Stirzaker and Both, 1989).

Extensive effort has gone into revealing the amino acid regions essential for ER retention. Two regions, aa 51-61 and aa 62-111, were found to be necessary for retention, but neither was sufficient alone (Poruchynsky and Atkinson, 1988). The H2 signal peptide was first found to be necessary (Stirzaker and Both, 1989) and later also sufficient together with aa 64-111 (Stirzaker et al., 1990). The most recent finding using site-specific mutagenesis and transfection experiments was that 31 residues of the final NH_2-terminus and H2 signal sequence were needed for retention (Maass and Atkinson, 1994). Interestingly, point mutations in amino acid sites Ile-59, Thr-60 and Gly-61 resulted in secretion of the molecule (Maass and Atkinson, 1994).

Through which part VP7 is bound to the ER membrane is not known. The signal peptide cannot function as an anchor, since it is rapidly cleaved (Stirzaker and Both, 1989), and the retention sequences did not promote membrane binding (Clarke et al., 1995). Clarke and coworkers (1995) suggested that the last 36 residues of VP7 might be present in the membrane or translocation pore, possibly with the C-terminus protruding into the cytoplasm, since these residues contributed to, but did not account for, membrane binding. Cross-linking analyses after sucrose gradient sedimentation have revealed the presence of oligomers of VP7 and NSP4 in rotavirus SA-11-infected MA104 cells (Maass and Atkinson, 1990). VP7 formed predominantly dimers and NSP4 formed tetramers. Complexes of VP4, NSP4 and VP7 were also found which were suggested to represent sites on the ER membrane that participate in budding of the single-shelled particles.

The importance of free Ca^{2+} in the stability of the outer membrane is well established. Studies with rVP7 protein in herpes simplex vector has given indirect evidence that VP7 binds Ca^{2+} without the presence of other rotavirus proteins (Dormitzer and Greenberg, 1992). An epitope of a neutralizing mAb was lost by Ca^{2+}-chelation with EGTA (ethylene glycol tetraacetic acid) suggesting that a conformational change of VP7 had occurred. The amounts of Ca^{2+} needed to stabilize the outer capsid of the virus have been shown to be strain-dependent (Ruiz et al., 1996). When mutant viruses selected for growth at low Ca^{2+} were sequenced, two prolines (aa 75 and / or 279) were found to be changed (Gajardo et al., 1997). The latter residue was suggested to be important for Ca^{2+}–binding of VP7, since the region was rich in oxygenated residues and since this proline was conserved in all group A VP7 sequences.

The glycosylation of proteins has been shown to be important for virus assembly in the enveloped viruses. In rotavirus, glycosylation of VP7 appeared to be prerequisite for disulphide bridge formation, since nonglycosylated VP7 did not interact with protein disulphide isomerase (Mirazimi and Svensson, 1998). Tunicamycin or a combination of tunicamycin and brefeldin A treatment caused misfolding of the VP7 protein and led to interdisulphide bond aggregation (Mirazimi and Svensson, 1998). A chimera of VP7-influenza haemagglutinin that was secreted and remained anchored in the plasma membrane was found to have a higher immunogenicity than intracellular rVP7 (Andrew et al., 1990). The membrane anchoring was shown for some reason to be essential for enhanced reactivity, since it was eliminated by triton-x treatment (Dormitzer et al., 1994).

Neutralizing epitopes. The major serotype-specific neutralization sites have been associated with the VP7 protein of rotaviruses. The four most common serotypes among human rotaviruses, G1-G4, were detected in 1978 (Flewett et al., 1978; Thouless et al., 1978; Zissis and Lambert, 1978). Plaque neutralization (Matsuno et al., 1977; Urasawa et al., 1982; Sonza et al., 1984) or fluorescent focus neutralization assays (Beards et al., 1980) were used for serotyping. In EIA (Thouless et al., 1982) or SPIEM (solid phase immuno-EM; Gerna et al., 1984) tests cross-absorbed antisera had to be used, which limited the usage of these techniques. Research in this field was increased, when serotype-specific mAbs could be produced (Greenberg et al., 1983b; Coulson et al., 1985).

Analyses of the neutralizing epitopes on VP7 have been performed by mAb tests combined with comparisons of the VP7 amino acid sequences. The VP7 sequences of different serotypes revealed extensive sequence conservation among serotypes, which was suggested to reflect structural and functional constraints necessary to preserve the architecture of the VP7 protein (Gunn et al., 1985). In all, nine variable regions, VR1-VR9 (aa 9-20, 25-32, 37-53, 65-76, 87-100, 119-132, 141-150, 208-224, 235-242, respectively) were found by sequence comparison (Green et al., 1989). The first three regions were regarded as unlikely to possess antigenic properties due to their hydrophobicity (Gunn et al., 1985), but the clusters of amino acid differences that were located in hydrophilic regions were regarded as potential antigenic sites. Gorziglia and coworkers (1986a) defined more or less the same variable hydrophilic regions on VP7: A (aa 39-50), B (aa 65-75), C (aa 87-101), D (aa 120-130), E (aa 146-149), F (aa 208-221) and G (aa 236-242). Region A had already been predicted to be a possible serotype-specific antigenic site after sequence comparison with VP7 genes from four rotavirus strains (Glass et al., 1985). It was also found that the predicted serotype of a rotavirus isolate by sequence analysis of regions A and C agreed with the result obtained by

serotyping it with mAbs (Green et al., 1988). The overall sequence identity among isolates of one serotype had been demonstrated by RNA-RNA hybridization of VP7-coding genes (Midthun et al., 1987). Comparisons of the deduced amino acid sequences among human and animal serotype G3 rotavirus strains showed overall sequence identities of 85% or higher (Nishikawa et al., 1989b).

Extensive studies using sequence analysis of antigenic mutants selected with mAbs have been performed in rotavirus isolates of the most common serotypes. Most of the results have been gathered in Figure 5. The major serotype-specific antigenic sites A (aa 87-96), B (aa 145-150) and C (aa 211-223) were reported by Dyall-Smith, 1986 (1986) with SA-11 escape mutants. The corresponding mutations were concentrated at the three antigenic sites independent of virus serotype, especially mutations at amino acid sites 94 and 96 in region A and at site 213 in region C were commonly found (Dyall-Smith et al., 1986; Mackow et al., 1988a; Taniguchi et al., 1988a; Nishikawa et al., 1989b; Coulson and Kirkwood, 1991; Kobayashi et al., 1991; Dunn et al., 1993; Kirkwood et al., 1993; Ciarlet et al., 1994; Lazdins et al., 1995; Coulson, 1996). Results from several different studies indicate that the three-dimensional folding of the native protein is such that regions A and C are in close proximity (Dyall-Smith et al., 1986; Taniguchi et al., 1988a; Kobayashi et al., 1991). The cross-reactive epitopes have also been localized in region A, e.g. at amino acid site 94 (Mackow et al., 1988a; Taniguchi et al., 1988a). Hoshino and coworkers (1994) found that aa 94 and 96 in region A and aa 213 in region C were involved in cross-reactive neutralization. Serotype-specific and cross-reactive neutralization epitopes were suggested to overlap operationally and constitute a single antigenic site (Morita et al., 1988; Taniguchi et al., 1988a). Neutralization by mAbs has also been shown to be affected by newly produced glycosylation sites in escape mutants (Kirkwood et al., 1993; Lazdins et al., 1995).

Monotypes. The antigenic differences shown by mAbs within one serotype divide strains into different monotypes or subtypes. Two distinct subtypes 4A and 4B of human rotavirus serotype G4 (Gerna et al., 1988) and three monotypes 1a, 1b and 1c of serotype G1 (Coulson and Kirkwood, 1991) were identified using neutralizing mAbs directed to VP7. Existence of G2 monotypes had also been suggested (Ward et al., 1991). Amino acids involved in distinguishing between monotypes of serotypes G2 and G4 were analysed (Coulson, 1996). Differences in amino acid sequences between G2 strains at positions 147 (region B), 213 and 217 (region C) correlated with the loss of mAb reactivity, aa 213 being of greatest importance. Antigenic region A (aa 94 and 96) was found to be most important for G4-subtype differentiaton. In serotype G1 a particular amino acid substitution at residue 94 in region A (Asn-Ser / Thr) correlated with monotype designation (Diwakarla and Palombo, 1999).

G serotype	A									B										C										References
	87								98			145				150						211					223					
G1			94			97																211			213						(Taniguchi et al., 1988a)
			94										147	148											213					291	(Coulson and Kirkwood, 1991)
			94							104	145							201							213	217	221			291	(Kobayashi et al., 1991)
G2			94														190			208					213					291	(Dunn et al., 1993)
			94	96																					213						(Lazdins et al., 1995)
													147												213	217					(Coulson, 1996)
G3			94	96									147									211			213						(Dyall-Smith et al., 1986)
			94	96	97		99															211	212								(Mackow et al., 1988a)
			94	96																							221				(Nishikawa et al., 1989b)
				96										148								211						238		264	(Lazdins et al., 1995)
G4			94	96																											(Coulson, 1996)
G9		91	94	96	97																				213				242		(Kirkwood et al,, 1993)
G11	87	91		96																											(Ciarlet et al., 1994)

Figure 5. *Location of amino acid changes in escape mutants within the neutralizing epitopes A, B and C on VP7.*

Genetic variation in G serotypes. Genetic variation in G serotypes has been studied using sequence analysis of human and animal rotavirus isolates. Xin and coworkers (1993) found an overall identity of more than 92% in nucleic acids and 96% in amino acids among Chinese and Japanese serotype G1 rotavirus VP7 sequences. The strains could be divided into three groups (subtypes); A, B and intermediate. The amino acid changes in the neutralization sites between the groups occurred most frequently at position aa 94 and 97 in region A, aa 147 and 149 in region B and aa 217 and 218 in region C. Analysis of G1 segment 9 sequences from vaccine failures revealed four distinct lineages (Jin et al., 1996). G1-specific neutralizing mAbs classified lineage II isolates as monotype G1a and lineage I isolates as monotype G1b (Diwakarla and Palombo, 1999). The sequencing analysis of Australian serotype G4 rotaviruses revealed sequence conservation in the neutralization epitopes (Palombo et al., 1993). A Thr-Asn change in region A at aa 96 affected mAb binding, but it did not result in complete subtype alteration.

Nonstructural proteins of rotaviruses

NSP1

NSP1 (NS53, NCVP2) is coded by gene segment 5 of SA-11 rotavirus. It is transcribed early, and the transcription is independent of protein synthesis in rotavirus-infected cells (Johnson and McCrae, 1989). Low amounts of NSP1 were found in the earliest replicative intermediate particles, precores (VP1, VP3, NSP1, NSP2 and NSP3), but not in the core particles, when replicative intermediate particles purified from virus-infected cells were analysed (Gallegos and Patton, 1989). In cultured cells large amounts of NSP1 have been detected in the cytosol and in association with the cytoskeletal matrix (Hua et al., 1994), where it showed a punctate and filamentous distribution. Experiments with deletion mutants indicated that the intracellular localization domain resided between aa 84 and 176 of NSP1, since the mutant NSP1-D82-476 accumulated in association with the nucleus, while the mutant NSP1-D177-476 showed a normal staining pattern (Hua et al., 1994).

The sequence analysis of gene 5 revealed that NSP1 had two regions with an arrangement of cysteines and histidines characteristic of zinc fingers (Bremont et al., 1987; Mitchell and Both, 1990b). The first motif contained amino acid residues 42-79 and the second motif residues 315-328. The first cysteine of the latter motif was found only in bovine RF and UK strains, so it is unlikely that a zinc finger common to all NSP1 sequences would be located in that part of the protein (Mitchell and Both, 1990b; Hua et al., 1993). The amino-terminal half of NSP1 has been shown to display conserved cysteines and histidines among all rotavirus strains, although the entire sequence shows

extensive sequence diversity (Mitchell and Both, 1990b), unlike other nonstructural proteins. The first zinc finger motif has also been found in group C rotaviruses (Bremont et al., 1993; Hua et al., 1993); this motif may form one or two fingers. NSP1 has three basic regions (aa 10-39, 79-93 and 111-126), in which the basic charge is conserved among rotaviruses.

Zinc finger proteins usually possess an RNA-binding capacity, and NSP1 has also been shown to recognize ssRNA in a nonspecific way, but with high affinity, in a gel retardation assay with rNSP1 (Brottier et al., 1992). In experiments with wild and mutant forms of NSP1, it exhibited affinity to all 11 rotaviral mRNAs from which it recognized an element near the 5'-end (Hua et al., 1994). The RNA-binding domain was found to localize in the aminoterminal part (within region aa 1-81), which indicated that the zinc finger indeed played a role in RNA binding.

The NSP1 sequences of different viruses exhibited in some cases identities as low as 36-38% (Hua et al., 1993). The lengths of the gene segments and NSP1 proteins have also been shown to vary (1564-1611 nt, 486-495 aa). Alignment of 17 NSP1 sequences revealed that only 88 amino acid positions (17.6%) were absolutely conserved among the rotavirus strains (Xu et al., 1994). The secondary structure predictions, however, showed high levels of conservation. Conserved stem-loop structures in the 5'- and 3'-ends of NSP1 mRNA have been proposed, but the RNA-folding programs did not predict that the 5'- and 3'-ends could interact (Hua et al., 1993). Dunn and coworkers (1994) showed by phylogenetic analysis that in clustering of NSP1 sequences according to species origin, only human and porcine strains clustered together. Recently, one feline rotavirus sequence was reported to show relatedness with those of bovine rotavirus strains (Kojima et al., 1996a).

Both the early *in vivo* and *in vitro* genetic reassortment studies showed that gene 5 did not segregate randomly and that it may enhance replication and be important in host range restriction (Graham et al., 1987; Broome et al., 1993; Kobayashi et al., 1995). Not much confirmation for this hypothesis has been achieved in recent studies, in which animals have been infected with reassortant rotaviruses (Bridger et al., 1998; Ciarlet et al., 1998). A reassortant virus with gene 5 from bovine rotavirus and the other genes from porcine virus was given to pigs. The reassortant virus replicated as well as the porcine virus, not as poorly as bovine rotavirus, in pigs (Bridger et al., 1998). Simian RRV has been found to be the only nonlapine virus that replicates well in rabbits and can spread horizontally (Ciarlet et al., 1998, 2000a). Experiments in which rabbits have been challenged with reassortants between simian RRV and human rotaviruses did not suggest that NSP1 would play a role in host range restriction. Instead, according

to the results obtained VP7 and to a lesser extent VP4 might be important in this respect, in contrast to Broome's and coworkers results with mice (1993). The VP7 and NSP1 sequences of RRV and lapine rotavirus ALA strain showed a high degree of amino acid identity with each other, 96-97% and 85-88%, respectively. The fact that RRV replicated well in rabbits and had similar VP7 and NSP1 sequences was suggested to reflect the importance of these proteins in host range restriction (Ciarlet et al., 2000a). The genetic analysis of NSP1 genes of rotaviruses (M37, ST3, RV3 and 1076) isolated from neonates with asymptomatic infection revealed that the strains did not carry a common NSP1 gene (Palombo and Bishop, 1994).

The function of NSP1 is currently not known, and the results from studies characterizing mutants with rearranged NSP1 genes have been somewhat surprising (Hua and Patton, 1994; Taniguchi et al., 1996; Okada et al., 1999). The carboxyl-half of it was not required for virus replication, and the capacity of binding to the cytoskeleton also remained (Hua and Patton, 1994). In another experiment two mutants of NSP1, one lacking the zinc finger, were found to replicate well in cultured cells, although one of the mutants produced very small plaques (Taniguchi et al., 1996). The zinc finger region of NSP1 was also not considered essential for genome segment reassortment with heterologous virus (Okada et al., 1999).

NSP2

NSP2 is a basic protein coded by gene segment 8 of SA-11 (Both et al., 1982). It has been shown to localize in the viroplasms (Petrie et al., 1984), which are proposed sites of genome replication. NSP2 has been suggested to play a role in RNA replication or packaging, since tsE mutant, which was found to map on gene 8 (Gombold et al., 1985), showed a large fraction of empty particles (Ramig and Petrie, 1984). TsE mutants lost the ability to synthesize dsRNA and also showed a reduction in synthesis of ssRNA (Chen et al., 1990). NSP2 was found in subviral particles with replicase activity together with VP1, VP2 and NSP3 (Helmberger-Jones and Patton, 1986) and in another study involving replicative intermediate precore, core and single-shelled particles in infected cells (Gallegos and Patton, 1989). A viral RNA-protein complex with replicase activity could be recovered with a mAb directed against NSP2 in an assay for the viral replicase (Aponte et al., 1996). This complex consisted of VP1, VP2, NSP2 and VP6. Only low amounts of NSP2 and NSP3 were found to be associated in membrane-enveloped viral intermediates together with VP1, VP2, VP4, VP6, VP7 and NSP4 (Poruchynsky and Atkinson, 1991).

Due to the presence of a helix-turn-helix motif in NSP2, it has been suggested to interact with nucleic acids (Estes and Cohen, 1989), and indeed it was shown to have affinity

for ssRNA and dsRNA in a sequence-nonspecific manner (Kattoura et al., 1992). Comparison of several mammalian virus NSP2 sequences revealed that they exhibited high levels of conservation in amino acids, ≥ 83% (Patton et al., 1993). The most basic and conserved region, aa 205-241, was suggested to be an RNA-binding domain. Conserved cysteins were found at sites aa 6, 8, 85 and 285 (Patton et al., 1993), which agreed with the presence of intra-chain disulphide bridges (Aponte et al., 1993). The first 75 nucleotides in the 5'-end and the last 28 nt in the 3'-end were found to be nearly identical among the mammalian rotavirus sequences and were suggested to be able to produce a stem double-loop structure (Patton et al., 1993). This panhandle structure may serve as a packaging signal for the assortment of NSP2 mRNA into replicase particles.

Aponte and coworkers (1993) detected oligomeric forms of NSP2 by mAbs against rNSP2 protein. Immunoprecipitation of the cross-linked lysates indicated that NSP2 multimers consisted of four or more molecules of the protein (Kattoura et al., 1994). Further studies showed that NSP2 proteins were bound to the 11 dsRNA genomic segments of rotavirus, particularly to partially replicated viral RNA (Aponte et al., 1996); however, only endogenous template was accepted. NSP2 was also shown to interact with VP1 in cross-linking studies (Kattoura et al., 1994). The complexes formed from NSP2, VP1 and mRNA were suggested to coordinate RNA packaging and the assembly of viral cores.

NSP2 was shown to interact with NSP5 (Poncet et al., 1997) and to colocalize with it in viroplasms in infected cells (Afrikanova et al., 1998). Coexpression of rNSP2 and rNSP5 resulted in viroplasm-like structures (Fabbretti et al., 1999), while expression of the proteins alone resulted in a diffuse cytoplasmic distribution (Kattoura et al., 1994; Fabbretti et al., 1999). Recently, NSP2 has been shown for the first time to have nucleoside triphosphatase activity (Taraporewala et al., 1999). Recombinant NSP2 could hydrolyse all four NTPs in a nonspecific manner in the presence of $MgCl_2$. Taraporewala and coworkers (1999) obtained evidence that hydrolysis of NTP resulted in the covalent linkage of the γ-phosphate to rNSP2, although earlier results failed to show any kinase activity of NSP2 (Aponte et al., 1996). *In vivo* the phosphorylation was shown to be transient, which was explained by the possible transfer of the γ-phosphate from NSP2 to NSP5 (Taraporewala et al., 1999).

Evidence has been obtained that NSP2 could destabilize DNA-RNA and RNA-RNA duplexes, similarly to sigmaNS protein of reovirus and ssDNA-binding proteins (Taraporewala and Patton, 2000). Recent results also indicate that octameric forms of NPS2 could be functional in the binding of RNA and ADP and that the protein may be

able to undergo ligand-induced conformational changes (Schuck et al., 2000).

NSP3

NSP3 protein is coded by segment 7 of rotavirus SA-11. Like NSP2, it was found in viral intermediate and replicase particles in infected cells (Helmberger-Jones and Patton, 1986; Gallegos and Patton, 1989) and tiny amounts in enveloped intermediate particles (Poruchynsky and Atkinson, 1991). NSP3 coding gene is one of the early genes, and its transcription is independent of protein synthesis (Johnson and McCrae, 1989). NSP3 is a slightly acidic protein (Both et al., 1984a; Ward et al., 1984) and has a relatively conserved amino acid sequence with identity of over 75% among mammalian rotaviruses (Rao et al., 1995). The lengths of the 3'-noncoding regions varied among rotaviruses, but a stretch of 80 nt in the 3'-UTR was highly conserved in the NSP3 gene among the 13 rotavirus strains compared (Rao et al., 1995).

NSP3 is also an RNA-binding protein (Boyle and Holmes, 1986) that could bind to all 11 viral mRNAs in a sequence-specific manner (Poncet et al., 1993, 1994). NSP3 was able to recognize the conserved sequence AUGUGACC of the 3'-end of group A rotavirus mRNAs, while NSP3 of group C rotaviruses recognized the sequence AUGUGGCU of the 5'-end of group C rotavirus mRNAs (Poncet et al., 1994). The minimal RNA sequence required for binding of NSP3 of group A rotaviruses was GACC.

NSP3 protein was found in homo-oligomers in insect cells, in SA-11-infected MA104 cells and in cell-free translation reactions (Mattion et al., 1992). Oligomers could be recognized by mAbs directed to rNSP3 (Aponte et al., 1993), and NSP3 has been shown to associate with itself in a yeast-two-hybrid system (Gonzalez et al., 1998). Both monomers and multimers could bind mRNA (Poncet et al., 1993). One NSP3 dimer could bind one molecule of mRNA (Piron et al., 1999). The dimerization site of NSP3 is locatee in the middle of the protein, aa 150-206, determined in the yeast-two-hybrid system, and dimerization of the protein has been shown to be important for strong RNA binding (Piron et al., 1999). The hydrophobic heptad repeats identified from position aa 181-236 have been implicated to be involved in oligomerization (Mattion et al., 1992).

A conserved basic region identified from amino acid position 81-150 in the N-terminal half of the protein has been proposed as the RNA-binding domain of NSP3 (Mattion et al., 1992; Rao et al., 1995). The basic region has also been found to be 40% conserved in NSP3 of group C rotavirus (Mattion et al., 1992). A consensus sequence (I/L)XXM(I/

L)(S/T)XXG for ssRNA-binding function identified in NS2 of orbiviruses and sigmaNS of reoviruses (van-Staden et al., 1991) was found to be conserved in NSP3 (aa 104-112), with the exception of the Met residue. Deletion mutation and point mutation experiments of NSP3 have indicated that the RNA-binding domain lies between amino acids 4 and 149 (Piron et al., 1999).

While the N-terminal part of NSP3 is involved in binding of viral mRNA, the C-terminal part can bind human eukaryotic initiation factor eIF4GI, which was found in a two hybrid system in yeast and confirmed by co-immunoprecipitation in rotavirus-infected cells (Piron et al., 1998, 1999). The results of Piron and coworkers (1998) indicated that NSP3 could take the place of poly A-binding protein (PABP) and shut off cellular protein synthesis. The position of NSP3 would allow viral mRNAs and cap-binding protein eIF4E to come close together and ensure efficient reinitiation of translation. This type of closed-loop model of mRNA translation is used by certain histone mRNAs that lack the polyA-tail (Gallie et al., 1996; Wang et al., 1996).

NSP4

NSP4 (NS28, NCVP5), the product of gene 10 of SA-11, is the most studied nonstructural protein of rotavirus. NSP4 was found to be glycosylated (Arias et al., 1982; Ericson et al., 1983). The carbohydrates were of a high-mannose type, $Man_9GlcNAc$ and $Man_8GlcNAc$ (Both et al., 1983b; Kabcenell and Atkinson, 1985). Only two potential glycosylation sites were located in the N-terminal hydrophobic region, which was a putative signal peptide, but which was not cleaved. When glycosylation of NSP4 was inhibited by tunicamycin, enveloped intermediate particles were found to accumulate in the ER, indicating that NSP4 was involved in assembly of the outer capsid (Petrie et al., 1983). NSP4 was shown to locate in the ER (Petrie et al., 1984) as an integral membrane protein (Kabcenell and Atkinson, 1985). Topographical studies indicated that a large C-terminal domain of NSP4 was exposed on the cytoplasmic side of the ER (Chan et al., 1988; Bergmann et al., 1989). Results from cell-free translation of NSP4 in the presence of dog pancreatic microsomes indicated that region aa 86-175 may be located on the cytoplasmic side (Chan et al., 1988). An even longer cytoplasmic domain (aa 44-175) was proposed by Bergmann et al. (1989), based on results obtained from comparison of glycosylation and proteolysis of NSP4 and its mutant forms. Only the second of the three hydrophobic domains of NSP4 was suggested to span the membrane, leaving the N-terminus and the first hydrophobic domain in the ER lumen (Bergmann et al., 1989). The third hydrophobic domain may be protected from proteases for reasons other than being integrated in the membrane, possibly due to multimerization of NSP4.

NSP4 has been suggested to help the single-shelled particles to bud through the ER membrane during morphogenesis. Support for this hypothesis was obtained when specific binding between purified single-shelled virus particles and RER membranes from rotavirus-infected cells was observed (Au et al., 1989). Recombinant NSP4 in insect cell membranes could also bind the single-shelled viruses. Complexes of VP4, VP7 and NSP4 have also been found in rotavirus-infected cells (Maass and Atkinson, 1990). Their association may represent sites on the ER membrane that participate in budding of the single-shelled particles into the lumen of the ER, where maturation to double-shelled particles occurs. The purified enveloped intermediate particles contained NSP4 together with the structural proteins VP1, VP2, VP4, VP6 and VP7 (Poruchynsky and Atkinson, 1991).

The domain on NSP4 responsible for binding to single-shelled particles was suggested to be the C-terminus of the protein, since an mAb shown to interact with the C-terminus and proteases known to cleave it reduced the binding (Au et al., 1989). The binding results were confirmed in a vaccinia recombinant system (Meyer et al., 1989), and the presence of Ca^{2+} and Mg^{2+} was found to enhance the binding, which had a dissociation constant of $5x10^{-11}$ M. The C-terminal Met has been shown to be essential for ligand binding by site-specific modifications of rNSP4 (Taylor et al., 1993). N-terminally truncated forms retained full binding activity (Taylor et al., 1993), but deletion of aa 161-175 abolished it (Au et al., 1993). Olivo and coworkers (1995) demonstrated that polymers of the synthetic peptide containing aa 160-169 of NSP4 aggregated single-shelled particles. The immature inner capsid-binding domain of NSP4 was located within the C-terminal 20 aa when a functional assay for capsid particle binding and biochemical and CD spectroscopic studies were combined (Taylor et al., 1996). Limited proteolysis and mass spectrometry analyses confirmed that 17-20 aa from the extreme C-terminus were necessary and sufficient for ligand binding (O'Brien et al., 2000).

The cross-linking studies revealed that NSP4 functions as oligomeric forms, mainly as tetramers (Maass and Atkinson, 1990). A recombinant deletion mutant lacking the first 85 aa could also form tetramers (Taylor et al., 1993). A region adopting an a-helical coiled-coil structure was suggested to mediate the oligomerization (Taylor et al., 1996). In the present model four flexible regions of 28 aa would be presented by a protease-resistant coiled-coil tetramerization domain, with only about the last 20 aa of each peptide interacting with the single-shelled particles (Taylor et al., 1996; O'Brien et al., 2000). The results implied cooperative bindings with one ligand molecule facilitating binding of the others to the same receptor (O'Brien et al., 2000). VP6 on single-shelled particles was suggested to interact with NSP4 (Au et al., 1989; Meyer et al., 1989). Later the spike protein VP4 was also shown to bind NSP4 (Au et al., 1993). The region aa 112-148 of NSP4 was critical for VP4-NSP4 binding.

The role of calcium was shown to be essential in the budding process and morphogenesis, since membrane-enveloped intermediates accumulated in calcium-depleted rotavirus-infected cells (Poruchynsky et al., 1991). Altered N-linked glycosylation and carbohydrate processing of NSP4 and especially VP7 was found. Inhibition of the disulphide bridge formation by DTT (dithiothreitol) also resulted in the accumulation of the enveloped intermediates (Svensson et al., 1994). Only VP7 was affected, since the mAb epitopes of VP7 disappeared, but not those of VP4 or NSP4. The concentration of calcium was found to increase nearly fivefold in infected insect cells that expressed NSP4 (Tian et al., 1994). Treatment of infected cells with thapsigargin, an inhibitor of the calcium-ATPase of the ER, also interfered with the glycosylation of NSP4 and VP7 and resulted in the accumulation of intermediate particles (Michelangeli et al., 1995). Exogenous NSP4 was found to affect the permeability of the ER membrane through the activation of phospholipase C, but the mechanism by which endogenously expressed NSP4 increased the calcium level appeared to be unrelated to phospholipase C (Tian et al., 1995).

Tian and coworkers (1995) also found that the synthetic peptide of 22 aa corresponding to aa 114–135 of NSP4 resulted in calcium mobilization in cells. Purified simian rotavirus NSP4 or the synthetic peptide was shown to induce diarrhoea in young CD1 mice (Ball et al., 1996). NSP4 was suggested to act as a viral enterotoxin that would trigger a signal transduction pathway. The NSP4 114-135 peptide was shown to potentiate chloride secretion by a calcium-dependent signaling pathway and alter intestinal epithelial transport. Recently, a fusion protein containing aa 86-175 of murine NSP4 expressed in *E. coli* was demonstrated to cause diarrhoea in the majority of young CD1 mice (Horie et al., 1999). Results obtained from a liposome leakage assay with fluorescent marker indicated that NSP4 and the synthetic peptide possessed membrane destabilization activity (Tian et al., 1996). A model for removal of the transient envelope by NSP4 during rotavirus morphogenesis was proposed. In this model, calcium is released from the ER concomitant with the budding process. The membrane-destabilizing activity was shown to be associated with the region aa 48-91, that includes a potential cationic amphipathic helix (Browne et al., 2000). Calcium mobilization by NSP4 was also shown to occur also in an *in vitro* cell line model (HT-29) for the human intestine through receptor-mediated phospholipase C activation and inositol 1,4,5-triphosphate production (Dong et al., 1997). In a dual-recombinant vaccinia system NSP4 was expressed in monkey kidney epithelial cells, which resulted in loss of plasma membrane integrity (Newton et al., 1997). Analysis of NSP4 deletion mutants indicated that a membrane-proximal region within the cytoplasmic domain (a loop between the second and third hydrophobic domains) mediates the cytotoxicity.

The amino acid sequences of NSP4 among mammalian rotaviruses could be clustered

into three genetic groups (Cunliffe et al., 1997; Horie et al., 1997a). Recently, the fourth genetic group was established (Horie et al., 1999; Ciarlet et al., 2000b) which contains murine NSP4 sequences that show an identity of only about 60% with the other sequences (Ciarlet et al., 2000b). The human NSP4 sequences clustered into two main groups, so that NSP4A included most VP6 sg I short e-type rotaviruses and NSP4B included sg II long e-type rotaviruses (Kirkwood et al., 1999). No correlation between VP6 subgroups and NSP4 groups was, however, found, when NSP4 sequences and subgroups of different animal species were compared (Ciarlet et al., 2000b). Feline rotaviruslike human Au-1 rotavirus NSP4 clustered into group NSP4C together with feline and canine NSP4 sequences (Horie et al., 1997a). The toxic peptide region exhibited amino acid variation at its carboxy terminus (aa 135-141) both within and between genetic groups (Cunliffe et al., 1997; Horie et al., 1997a).

The role of toxic peptide in causing diarrhoea has remained unclear so far. Kirkwood and coworkers (1996) found one amino acid difference, aa 135 (Ile/Val), between asymptomatic and symptomatic viruses, although VP4 and VP7 sequences also showed several differences. In another study NSP4 sequences of virulent and attenuated virus strains revealed one amino acid change at position 45 (Ward et al., 1997). This Ala-Thr substitution could be found both in attenuated and wild type human group A rotavirus strains, and thus it did not explain the attenuation. Studies with cystic fibrosis transmembrane conductance regulator knockout mice also failed to show consistent or significant changes in NSP4 sequences between virulent or attenuated viruses (Angel et al., 1998). On the other hand, Zhang and coworkers (1998) were able to show association of mutations in NSP4 and altered virus virulence. Virulent porcine OSU strains increased intracellular calcium levels, whereas avirulent OSU strains had little effect. Mutated virulent OSU NSP4 with deletions or substitutions in the region aa 131-140 lost its ability to increase intracellular calcium levels and to induce diarrhoea in neonatal mice. Structural changes in the region aa 131-140 were suggested to affect pathogenesis. Results from infecting gnotobiotic piglets with reassortant viruses indicated that several rotavirus proteins (VP3, VP4, VP7 and NSP4) may be associated with virulence (Hoshino et al., 1995).

The most recent finding on the NSP4 enterotoxin peptide is that it is secreted from rotavirus-infected cells (Zhang et al., 2000; Levy et al., 2000). The secreted cleavage product of NSP4 represented aa 112-175. Purified cleavage product of NSP4 (aa 112-175) was shown to increase intracellular calcium mobilization in intestinal cells, and the peptide expressed in insect cells was able to cause diarrhoea in neonatal mice (Levy et al., 2000). Cleavage of NSP4 was observed only when infections were carried out in the presence of trypsin.

NSP5

NSP5 is a nonstructural protein coded by segment 11 in the rotavirus SA-11 strain. The protein is hydrophilic and rich in serine and threonine (Imai et al., 1983b; Ward et al., 1985). It is the only rotavirus segment, in which more than one ORF has been found (Mitchell and Both, 1988). It has two overlapping ORFs; the longer codes for the NSP5 protein of 198 aa and the out-of-phase ORF (+1) codes for the protein NSP6 (formerly NSP5a) of 92 aa. NSP5 was localized to viroplasmic inclusions using colloidal gold in EM analysis (Petrie et al., 1984). Monospecific antiserum to rNSP5 also detected discrete foci, viroplasmic inclusions, in the cytoplasms of infected monkey kidney cells experiments (Welch et al., 1989). No NSP5 was found from purified virus particles, although NSP5 was assigned as a structural protein in some earlier reports (McCrae and McCorquodale, 1982a; Mason et al., 1983).

In addition to the 26-kDa product of NSP5, a 28-kDa product was shown to be related to it by protease mapping (Ericson et al., 1982). Both forms were shown to be phosphorylated (Welch et al., 1989). NSP5 was also found to be modified by addition of single O-linked residues of N-acetylglucosamine (Gonzalez and Burrone, 1991). It was suggested that NSP5 reversibly blocks sites of phosphorylation on NSP5, thereby regulating functioning of the protein.

Additional forms of NSP5, varying 32-34 kDa, were found (Afrikanova et al., 1996). Treatment with phosphatases of these different forms produced a single band of 26 kDa, indicating that the level of phosphorylation affected migration of the protein in gel (Afrikanova et al., 1996; Blackhall et al., 1997). The highest-molecular-weight forms of NSP5 were more heavily phosphorylated than the 26-kDa form (Poncet et al., 1997). The latter, however, still contained phosphate groups that were not removed by phosphatase A (Blackhall et al., 1997). The hyperphosphorylated forms (32-34 kDa) appeared to contain very little or no O-glycosidic residues (Afrikanova et al., 1996). In pulse-chase experiments the 26-kDa form was detected first, at 2 hours p.i., and then the 28- and 35-kDa forms 2 hours later (Blackhall et al., 1998). Controversial data about the phosphorylation sites on NSP5 have been reported; either the phosphates were found attached via Ser and Thr residues (Afrikanova et al., 1996), or only Ser residues were found to be phosphorylated (Blackhall et al., 1997).

Results suggesting the presence of autophosphorylation activity associated with NSP5 were obtained in *in vitro* experiments with rNSP5 expressed in *E. coli* (Afrikanova et al., 1996; Blackhall et al., 1997; Poncet et al., 1997). Despite the existence of autophosphorylation of NSP5, its fully phosphorylated form has not been detected in

expression systems. Since the localization of NSP5 in infected cells is different from that of the *in vitro* expressed rNSP5, correlation between the localization of NSP5 in viroplasms and its protein kinase activity has been proposed. The necessity of cellular or viral cofactors was suggested (Poncet et al., 1997). It is unclear whether cellular protein kinases also participate in NSP5 phosphorylation. Phosphorylation of NSP5 expressed in COS-7 cells was partially inhibited by protein phosphatase inhibitor staurosporine, whereas the autophosphorylation of the purified polypeptide was not (Blackhall et al., 1998).

The protein from the out-of-phase ORF of NSP6 was shown to be expressed in rotavirus-infected MA104 cells (Mattion et al., 1991), using monospecific sera prepared against NSP6. An interaction between NSP5 and NSP6 were shown in a yeast two-hybrid system (Gonzalez et al., 1998), and also dimers of NSP5 were detected (Poncet et al., 1997; Gonzalez et al., 1998). The multimerization site was located at the C-terminus of NSP5, which forms an a-helical structure (Torres-Vega et al., 2000). The binding site of NSP6 for NSP5 protein was found to overlap with the multimerization domain. Since phosphorylation was poor in mutants with deletion in the C-terminus, multimerization was proposed to be important for the activity of NSP5, and NSP6 was suggested to play a regulatory role in its self-association.

As previously stated, NSP5 was shown to interact with NSP2 (Poncet et al., 1997) and to form viroplasm-like structures with it (Fabbretti et al., 1999). In another report, NSP5 could be chemically cross-linked with VP1 and NSP2 in living cells (Afrikanova et al., 1998). In transient transfection assays, NSP5 phosphorylation was enhanced by coexpression of NSP2. A 33-aa N-terminal deletion mutant was shown to become phosphorylated, but to be insensitive to NSP2 activation, suggesting that NSP2 may bind to the N-terminus of NSP5 (Afrikanova et al., 1998). Both the N- and C-terminal domains of NSP5 were found to be essential for viroplasm formation when NSP5 deletion mutants were expressed in combination with NSP2 (Fabbretti et al., 1999). Hyperphosphorylation of NSP5 was found to be necessary, but not sufficient for viroplasm formation.

Genome rearrangements of gene 11. Several genome rearrangements of gene 11 have been reported, and they group roughly into two types of rearrangements: either a partial duplication of the ORF region or an AT-rich insertion in the 3'-noncoding region. The longer segment 11 has been easy to visualize by its slower migration in gel, while it normally is the fastest moving RNA segment of rotavirus. The RNA patterns occurring with rearranged segment 11 are called either short or super-short electropherotypes (e-type) in contrast to the normal long e-type. Observations common

to all rearrangements are that one complete ORF region is preserved and normal NSP5 protein appears to be produced.

Rearrangements with duplications contain a duplicated ORF region in a head-to-tail orientation following the normal ORF region, with the exception that it lacks bases from the 5' end including the AUG initiation codon and thus is non-coding. This type of rearrangement was found in swine rotaviruses (Gonzalez et al., 1989; Mattion et al., 1990), the rabbit rotavirus Alabama strain (Gorziglia et al., 1989), a bovine rotavirus (Scott et al., 1989), the human rotavirus strain Mc323 (Kojima et al., 1996b) and a rotavirus strain isolated from a chronically infected immunodeficient child (Palombo et al., 1998). The following mechanism for rearrangements has been proposed: rotavirus RNA-dependent RNA polymerase pauses during RNA synthesis due to the secondary structure of the template or other cause. The RNA polymerase dissociates from the template or slips back along it, reattaches to the same template without releasing its nascent transcript and reinitiates transcription to the normal 3'-end of the RNA (Gorziglia et al., 1989; Scott et al., 1989; Kojima et al., 1996b). Nearly identical sequence of six bases has been found adjacent to the reinitiation site and the recombination site in rearranged segment 11. The orientations of these direct repeats indicated that the rearrangement had occurred at the stage of plus-strand synthesis (Kojima et al., 1996b).

In porcine rotavirus, the supershort variant CC86 with partially duplicated segment 11 was able to produce larger plaques and overgrew the normal strain CN86 in coinfection (Mattion et al., 1990). A reassortant containing segment 11 of the supershort strain in the background of simian SA-11 also produced larger plaques (Chnaiderman et al., 1998).

The short e-types of human rotaviruses (strains RV-5, DS-1 and some Argentinian strains) represent the rearrangement type of gene 11 in which the 3'-noncoding region contains an AT-rich insert (Nuttall et al., 1989; Matsui et al., 1990; Giambiagi et al., 1994). This AT-rich region (148-158 bp) is highly homologous among the strains, although it is not similar to any other rotavirus gene sequence. The short e-types are stabile circulating strains that usually show VP6 subgroup specificity of sgI, VP7 serotype of G2 and VP4 serotype of P[4], although exceptions have been found.

Group C rotaviruses (pararotaviruses)

History

During the 1980's rotavirus researchers found rotaviruslike particles in the intestinal contents of piglets, which did not react with antisera to porcine, bovine or human rotaviruses (Saif et al., 1980). Gnotobiotic pigs orally exposed to viruses developed diarrhoea and shed the viruses. Villous enterocytes of the small intestines became infected as in rotavirus infection (Bohl et al., 1982; Chasey and Banks, 1986). Although the viruses had 11 RNA segments, their migration pattern in gel was different from that of the group A rotaviruses. They were named group C rotavirus and the previously known rotavirus as group A rotavirus (Snodgrass et al., 1984). The first human group C rotavirus was found a couple of years later (Rodger et al., 1982). Group C rotaviruses infect humans much more rarely than group A viruses (Dimitrov et al., 1983; Nicolas et al., 1983), although they are common in pigs and cattle.

The physicochemical characteristics of the group C rotaviruses are nearly identical to the group A rotaviruses. After centrifugation in the sucrose gradient, they showed a sedimentation coefficient equal to that of SA-11, but a slightly higher buoyant density was observed after isopycnic centrifugation in CsCl (Espejo et al., 1984). Minor differences in activation of group C polymerase have also been reported, which were suggested to result mainly from the differences in stability of outer shell membrane (Jashes et al., 1986). Group C particles tend to occur mostly as intact virions and not so often as single-shelled particles as group A rotaviruses.

Porcine group C rotavirus was adapted to serial propagation first in primary porcine kidney cells, using a high concentration of pancreatin and roller tubes (Terrett and Saif, 1987) and later in MA104 cells (Terrett et al., 1987; Saif et al., 1988). This porcine Cowden strain attained a virus titre of 10^7 immunofluorescence U / ml in continuous cell culture. The culture-passaged virus also retained its infectivity for gnotobiotic pigs. The porcine Cowden-AmC-1 strain has also been adapted to grow in diploid swine testicular cell line (ST cells) in the presence of trypsin (Welter et al., 1991). In addition to porcine viruses one bovine group C rotavirus, Shintoku, has been adapted to serial propagation in MA104 cells (Tsunemitsu et al., 1991). So far only one publication reporting serial propagation of human group C rotaviruses in a cell line (CaCo-2), has been published (Shinozaki et al., 1996). The infected cells showed no cytopathic changes. The virus was detected by immunofluorescence staining and by reverse passive haemagglutination assay.

The viral group C haemagglutinin was identified by using agglutinated human and

guinea pig erythrocytes (Svensson, 1992). Infection of ST cells with Cowden AmC-1 virus was prevented by neuraminidase treatment. Sialic acid was suggested to constitute an essential part of the cell receptor. A neutralizing mAb with haemagglutination inhibition activity has been produced (Nilsson and Svensson, 1995). It was shown to recognize an epitope located on the VP5* cleavage fragment of the VP4 protein.

Polypeptides

A complete polypeptide map for all the 11 group C rotavirus proteins is still not available, but in principle the same proteins are produced by group C as by group A rotaviruses. Six structural proteins have been detected ranging in molecular weight from 125 000 to 37 000 in the porcine virus (Bremont et al., 1988; Jiang et al., 1990). A mannose-rich glycosylated protein of 39 kDa was shown to be located in the outer virion shell and corresponded to VP7 protein. The 52-kDa and 33-kDa proteins found by Bremont and coworkers (1988) and Jiang and coworkers (1990), respectively, were actually the cleavage products of the 78-kDa VP4 spike protein (Nilsson et al., 1993). Four polypeptides (125, 93, 74 and 41 kDa) corresponding to VP1, VP2, VP3 and VP6 were located in the inner shell of group C rotavirus (Jiang et al., 1990). Polypeptides with approximately the same molecular weights (122, 98, 79 and 43 kDa) were found by Nilsson and coworkers (1993).

The nonstructural proteins of group C rotaviruses are still poorly characterized. Jiang and coworkers (1990) suggested that 39-kDa and 35-kDa proteins may correspond to the proteins NSP1 and NSP2 and the glycosylated 25-kDa protein may correspond to NSP5 of group A rotavirus. Nilsson and coworkers (1993) found nonstructural proteins with mw of 35, 24 and 20 kDa in SDS (sodium dodecyl sulphate)-PAGE analysis of ^{35}S-Met-labelled infected cell lysates. *In vitro* transcription and translation of genomic RNA from a porcine group C rotavirus revealed at least nine viral proteins, ranging from 93 kDa to 22 kDa (Jiang and Saif, 1992). NSP3 protein was expressed *in vitro* and in COS-1 cells (Langland et al., 1994). Minor amounts of a product with mw of 45 kDa and major amounts of products with mw of 38 kDa and 8 kDa were synthesized.

Genome

The fingerprint bands closest to the termini were found to be conserved across all of the genome segments of group C rotaviruses (Bridger et al., 1986). As with group A rotavirus NSP3, group C rotavirus NSP3 was also shown to recognize a specific RNA sequence AUGUGGCU that is conserved in the 3'-ends of group C rotavirus mRNAs (Poncet et al., 1994).

The main focus of group C rotavirus research has been on the identification and sequencing analysis of the RNA segments. Data concerning the porcine Cowden strain are shown in Table 3, since it is the most extensively studied group C rotavirus. Several RNA segments of the human Bristol (Cooke et al., 1991; Lambden et al., 1992; Fielding et al., 1994; Grice et al., 1994; Deng et al., 1995; Samarbaf-Zadeh et al., 1996; James et al., 1999) and bovine Shintoku strains (Jiang et al., 1992a; Jiang et al., 1993; Tsunemitsu et al., 1996; Jiang et al., 1999) have also been characterized. As the table shows sequence identities between group A and C rotavirus RNA segments are less than 50%. The phylogenetic analyses suggest that group A, B, and C rotaviruses have diverged at a constant rate from a common ancestor (James et al., 1999).

Nearly all RNA segments of human and porcine group C rotaviruses shared a high degree of nucleotide sequence homology when they were compared by cross-hybridization using radiolabelled ssRNA transcript probes (Qian et al., 1991c). The greatest sequence divergence was observed in NSP1-coding gene 7. A gene 6 (NSP3) cDNA probe of the Cowden strain was also reported to hybridize weakly with the corresponding genes of human and bovine group C rotaviruses (Jiang et al., 1992b).

Group A rotavirus versus group C rotavirus

Bremont and coworkers (1988) could find no cross-reactions between group A and group C rotaviruses by immunoblotting. MAbs to group C rotavirus VP6 did not react with group A or B rotaviruses (Ojeh et al., 1991). Certain mAbs to Cowden VP6, however, were demonstrated to cross-react with group A and group C rotaviruses (Tsunemitsu et al., 1992b). It has been suggested, however, that common epitopes would not be located in an immunodominant region. Seven regions in VP6 have been found in which amino acid sequence identity between group A and C rotaviruses ranges from 70% to 90%. Three of these regions (aa 95-113, 123-140 and 198-206) are hydrophilic and show structural homology (Jiang et al., 1992a). They have been suggested to contribute to potential common epitopes between distinct serogroups of rotaviruses.

Virtually all the functional parts of the group A rotavirus proteins were also found to be conserved in group C rotavirus, such as the cysteine-rich domain of NSP1 (Hua et al., 1993) and the RNA-binding site of NSP3 (Langland et al., 1994; Piron et al., 1999). The overall structure of the membrane-spanning NSP4 protein is also conserved, with the exception that it lacks one of the three N-terminal hydrophobic domains (Horie et al., 1997b). Another difference between the serogroups has been found in that NSP5 of group C rotavirus presented only a single large ORF; thus a protein corresponding to NSP6 of group A rotavirus was not produced (Lambden et al., 1992; Bremont et al.,

Table 3. *Group C rotavirus RNA segments and proteins (strain Cowden).*

RNA segment	Length (bp)	Protein	Length (aa)	MW	References	Identity with group A rotavirus (%)
1	3290	VP1	1082	125 000	(Bremont et al., 1992)	48
2	2655	VP2	873	101 036	(Bremont et al., 1992)	44
3	2246	VP4	692	81 306	(Bremont et al., 1992)	33
4	2162	VP3	736	83 156	(Bremont et al., 1992)	35
5	1352	VP6	395	44 614	(Bremont et al., 1990) (Cooke et al., 1992)	42
6	1348	NSP3	402	-	(Qian et al., 1991b)	22
7	1235	NSP1	393	46 400	(Bremont et al., 1993)	15
8	1063	VP7	332	37 300	(Qian et al., 1991a)	<30
9	995	NSP2	312	35 810	(Bremont et al., 1993)	37
10	693	NSP5	210	23 377	(Bremont et al., 1993)	45
11	613	NSP4	150	-	(Chang et al., 1999)	-

1993).

Coexpression of group A and C rotavirus rVP6 did not result in the formation of mixed trimers (Tosser et al., 1992). Empty single-shelled particles, however, were formed when group A rotavirus rVP2 and group C rVP6 proteins were coexpressed, although many of them had heterogeneous morphology (Tosser et al., 1992). Group C rVP6 was not able to restore the transcriptase activity of those single-shelled particle, but group A rVP6 could restore it (Kohli et al., 1993). Group A and C rotavirus VP6 proteins were also expressed in primary neurons and mammalian epithelial cells, using the Semliki Forest virus expression system (Nilsson et al., 1998). The VP6 proteins of group A and C rotaviruses showed different immunofluorescence patterns in BHK-21 cells; VP6 C displayed an intense punctate immunofluorescence pattern, while VP6 A showed a filamentous staining.

Serotypes and genetic diversity among group C rotaviruses

Genetic diversity also exists among group C rotaviruses, although on a much narrower scale than among group A rotaviruses. Under high-stringency conditions, group C rotavirus strains were divided into two groups in dot-blot hybridization assay when VP7-encoding gene 8 was used as a probe (Jiang et al., 1991). Porcine Cowden and bovine Shintoku strains were classified as different serotypes by two-way cross-neutralization tests, and the existence of a third serotype (for porcine HF strain) was suggested (Tsunemitsu et al., 1992a). The amino acid identity between the serotypes ranged from 70% to 75%, while it was 85-97% between Cowden-related strains that formed one serotype (Tsunemitsu et al., 1996).

All human group C rotaviruses analysed so far have belonged to the same serotype (Jiang et al., 1996). Evidence for further genetic clustering within the serotype has been reported in a Japanese study in which two Japanese rotavirus strains with different electropherotypes had a nucleic acid identity level of 95.6% (identity 96.7% in amino acids; Kuzuya et al., 1996). The existence of different VP4 serotypes has also been suggested (Fielding et al., 1994). VP4 sequences of human Bristol and porcine Cowden strains showed a nucleic acid identity of only 71% and the human strain had eight amino acids more than the porcine strain. Most of the nucleic acid differences between the strains were located in the VP8* cleavage product of the VP4 protein.

AIMS OF THE PRESENT STUDY

The aims of the study were to investigate at serological and molecular level the epidemiology of human rotaviruses encountered in Finnish children during an observation period of 17 years. The investigation included:

1. Characterization of the group C rotaviruses encountered and establishment of serological methods for group C rotavirus infections.

2. Detection and characterization of the genetic diversity among group A rotaviruses by electropherotyping, serotyping and sequence comparisons. In more detail, the aims were:

- To analyse the e-types and the prevalence of different G and P serotypes during the observation period

- To study the interrelationships between the circulating rotavirus strains

- To search for genome characteristics common for the predominant rotavirus strains among G1P[8] and G4P[8] e-types

MATERIALS AND METHODS

Patient samples. The study material consisted of rotavirus-positive stool samples determined by EM in the Department of Virology, University of Helsinki, (up to 1997) and in the Division of Virology, Helsinki University Central Hospital (year 1997). The samples studied were obtained during 1976-1997. Most of the samples were from children under 3 years of age who were hospitalized for acute gastroenteritis. The samples were mainly sent from three metropolitan hospitals: the municipal Aurora Hospital of Helsinki, Children's Hospital, and Jorvi Hospital. In a four year period (1987-1990) 44.4%, 37.6%, 7.7% and 10.3% of the samples were obtained from Aurora, Children's Hospital, Jorvi and other hospitals, respectively. The relative amounts of the samples sent from each hospital have recently changed due to hospital fusions. Those stool suspensions in which rotaviruses were detected by EM were stored at –20 °C. Only 86 stool suspensions had been left from the period of 1976-1980 (7, 3, 9, 45 and 22 samples per year, respectively), but from then on practically all rotavirus-positive suspensions were stored and served as material for this study.

Electron microscopy (EM). The faecal samples were suspended in a buffer containing 0.05 M Tris, 0.1 M NaCl, 1 mM $CaCl_2$, pH 7.4, (10% (w/v) suspension). Viruses were identified on grids negatively stained with 2% potassium phosphotungstate (pH 5.5-6.0) for EM (Jeol, JEM-100CX II, Tokyo, Japan).

Viruses and cell cultivation. Porcine group C rotavirus AmC-1 was a gift from L. Svensson (SIIDC, Karolinska Institute, Stockholm, Sweden), and its characteristics are those reported by Welter et al. (1991). The group C rotavirus that had been obtained from the American Type Culture Collection (UR-958; Cowden strain) was adapted by them to grow in cell culture. A virus stock was prepared by growing the viruses in ST cells for 2 days. The cells were frozen and thawed three times, centrifuged for 20 min (2500 rpm) and the supernatant was stored in aliquots at –70 °C. The virus stock contained viruses at a titre of about 10^7 pfu/ml when determined by infectious focus assay.

The ST (swine testicular) cell cultures were grown in Eagle's MEM (minimum essential medium) supplemented with nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, sodium bicarbonate and 10% fetal calf serum. The porcine group C AmC-1 rotavirus was pretreated with 10 µg/ml trypsin (Sigma) for 1 h at 37 °C before inoculation. The ST cells that were grown to confluence in roller tubes were washed with serum-free MEM and incubated for 30-60 min before virus inoculation in MEM alone. Virus at a dilution of 1:100 was allowed to adsorb onto cells for 1 h at 37 °C in a small liquid volume. The cells were grown in the presence of MEM supplemented as above but lacking fetal calf serum. The presence of 8 µg/ml trypsin was necessary for virus replication.

The simian group A rotavirus SA-11 that was used as a marker in PAGE was cultivated in foetal rhesus monkey kidney cell line, MA104 (Microbiological Associates, Bethesda, MD, USA). The cells were grown in Eagle's MEM supplemented with 10% fetal calf serum and 1 mM Hepes. The SA-11 rotavirus was also pretreated with 10 µg/ml trypsin, but cultivated in the presence of only 1 µg/ml trypsin.

RNA extraction and gel electrophoresis. For nucleic acid extraction, a 600-µl volume of stool suspension was used. The entire procedure was performed at room temperature. A volume of 100 µl saturated phenol and 100 µl chloroform-isoamyl alcohol (CHIS, 24:1) were combined with the sample and the tubes shaken vigorously for 2 min. After centrifugation for 3-5 min in an Eppendorf-centrifuge, the upper water phase was gathered. The phenol-CHIS extraction was repeated, and a third extraction with 200 µl CHIS alone was carried out. The volume was decreased by ethanol precipitation: 40 µl 3 M Na-acetate, pH 5.4 and 800 µl ice-cold ethanol were added, and after incubation for 10-20 min at –70 °C, the precipitate was pelleted by centrifugation for 20 min at 4 °C. The precipitate was vacuum-dried and suspended in 25-60 µl sample buffer (10% sucrose, 0.2% bromphenol blue in 25 mM Tris, 0.19 M glycine) depending on the virus amount estimated by EM.

Gel electrophoresis was run in a 7.5% polyacrylamide gel (Laemmli, 1970) in a 25-mM Tris, 0.19-M glycine buffer. Either a large (length of the gel: 12 cm, thickness 1.5 mm) or a small (length: 7 or 9 cm; thickness 0.75 mm; Mighty Small, Hoefer Scientific Instruments, San Fransisco, CA) electrophoresis machine was used. The electropherotypes of the samples were determined with the large gels, while the small gels were used mainly for coelectrophoresis. A 20-µl sample or SA-11 marker was added to a lane of the large gel and the electrophoresis was run at 90 V overnight for 17 h at room temperature. In the small gel 3-8 µl sample were added per lane and run for 1.5 h (7-cm gel) or 3 h (9-cm gel) at 250 V cooling with tap water. Since the RNA pattern of an e-type was somewhat different in small and large gels, the e-types could not be cross-compared.

Silver staining. The silver staining method was modified from that of Herring et al (1982). All the incubation steps were performed in a shaker at room temperature. The large gels were fixed in 10% ethanol, 0.1% acetic acid for 30 min and stained in 0.18% $AgNO_3$ for 30 min. The excess stain was removed by washing in distilled water for 7 min. The gels were then soaked in 3% NaOH with fresh formaldehyde (8 ml/l) until the bands appeared (max 20 min). The reaction was stopped in 5% acetic acid, 0.5% glycerol, and the gels photographed and dried.

Serotyping by EIA. A commercial set of mAbs for serotyping human rotaviruses was used (Serotec ROTA-MA, Sapporo, Japan). The mAbs were prepared against rotavirus strains KU (G1), S2 (G2), YO (G3) and ST3 (G4; Taniguchi et al., 1987b). The procedure included in the kit

was followed with some modifications. Microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with mAbs diluted in PBS (100 µl / well) and incubated overnight at 4 °C. The wells were washed three times with PBS (300 µl / well) and blocked with PBS, 0.05% Tween 20 (=PBST), 1% BSA (250 µl / well) at 4 °C overnight. The wells were washed twice as described and the samples (50 µl 10% stool suspension per well) diluted 1:4 in PBST-10% skimmed milk were added into each well and incubated at 4 °C overnight. After a washing step 50 µl anti-SA-11 hyperimmune rabbit serum (1:1000 in PBST-2.5% skimmed milk) were added to the wells and incubated for 1 h at 37 °C. The wells were washed and 50 µl peroxidase-conjugated anti-rabbit immunoglobulin (Dakopatts, Copenhagen, Denmark) diluted 1:2000 with PBST were added in each well. After incubation for 30 min at 37 °C the wells were washed four times with PBS and once with 0.1 M citrate-phosphate buffer, pH 5.0 for 1 min. As a substrate, 100 µl 1,2-phenylenediamine, dihydrochloride (Dakopatts, 2-4 tablets / 12 ml) in 0.1M citrate-phosphate buffer containing freshly added H_2O_2 (5 µl 30% H_2O_2 / 12 ml) were added to each well. The reaction was stopped after a 30-min incubation by addition of 25 µl 20% H_2SO_4 per well. The absorbance values were measured at a wavelength of 492 nm with Multiskan (Labsystems, Helsinki, Finland). A positive result to a specific serotype was assigned if the absorbance value was > 0.2 and higher than twice the value of any other serotype.

Neutralization test (NT). Virus (group C rotavirus AmC-1 or group A rotavirus SA-11) was pretreated with trypsin as described and the sera were incubated at 56 °C for 30 min. The confluent cell cultures grown in cell tubes were washed three times with Eagle's MEM. Equal amounts of virus (50 $TCID_{50}$ / tube) and sera (diluted in fourfold steps starting from a 1:5 dilution) were combined and incubated for 1 h at room temperature. The mixture was inoculated into cells (200 µl / tube) and adsorbed for 1 h at 37 °C. Cells were grown in serum-free growth medium (2 ml / tube) in the presence of trypsin (1 or 3 µg / ml for MA104 or ST cells, respectively). The cells were examined for the presence of CPE characterized by syncytia formation and subsequent cell lysis. The development of CPE was followed keenly before the presence of trypsin caused the cells to detach from the monolayers.

Immunofluorescence antibody test (IFAT). For the IFAT test, slides containing cells from which 5-10% were positive were prepared. Cells infected with virus were trypsinized after about 14 h, washed four times with PBS and diluted in 13 ml of the same buffer. Cell suspension (25 µl / well) was pipetted on 12-well microscope slides, allowed to air-dry and the cells fixed with ice-cold acetone for 7 min. The slides were stored at –70 °C. Serum samples were diluted in PBS in twofold steps for IgM detection and in fourfold steps for IgG detection beginning with a dilution of 1:4. Serum dilutions were pipetted into the wells (18 µl / well) and the slides incubated for 30 min at 37 °C in a wet chamber. After washing for three times with PBS and once with distilled water, the slides were gently dried. As a conjugate, fluorescein-conjugated rabbit immunoglobulins to human IgM (µ-chain; Dakopatts) and fluorescein-conjugated goat

antiserum to human IgG (Kallestad, Austin, TX, USA) were used in the IgM and IgG tests, respectively. The same amount of conjugate was added to each well, incubated for 30 min at 37 °C in a wet chamber and washed as before. After the slides were dried, mounting media was added and the slides were covered with coverslips.

Haemagglutination inhibition (HI). For the HI test the serum samples were treated with kaolin. Unheated serum (50 µl), 200 µl borate buffer, pH 9.0 and 250 µl 25% kaolin (Fluka) were combined and incubated for 20 min at room temperature with vigorous shaking every 5 min. After a 5-min centrifugation the supernatant was separated and used in the HI assay. Twofold dilutions of the serum in Dulbecco, 0.2% BSA (50 µl per well) were pipetted into a microtitre plate (round-bottomed). A crude antigen for HI was prepared as follows: ST cells infected with porcine group C rotavirus AmC-1 were ruptured by freezing and thawing three times and the cell debris was removed by centrifugation. The antigen (20 µl per well) was added to a microtitre plate and serum and antigen were incubated for 60 min at room temperature. Human O erythrocytes suspended in Dulbecco-BSA (0.5% suspension) were added to the wells (50 µl), and after 50-60 min the result was readable.

Antibody EIA. For antibody EIA assay, rabbit hyperimmune serum against group A rotavirus (SA-11) or group C rotavirus (AmC-1) was used for coating microtitre plates. A volume of 100 µl hyperimmune serum diluted in sodium bicarbonate buffer was added to each well and the plates incubated at 4 °C overnight. After washing six times with PBST (150 µl per well) a crude antigen prepared from either group A or group C rotavirus was added to each well (100 µl per well). An overnight incubation was followed by a washing step, three times 5 min with PBST and once for 1 min with distilled water. Patient sera diluted in PBST (dilution 1:50 and 1:200 for IgM and IgG, respectively) were added (100 µl) and incubated for 1 h at 37 °C. The procedure after the washing step was the same as in serotype-EIA, with the exception that the conjugate and substrate were added 100 µl per well and the reaction was stopped by addition of 1M H_2SO_4 (150 µl per well). The absorbance values were measured at a wavelength of 492 nm with Multiskan.

RNA extraction for RT-PCR. For gene amplification, RNA from rotavirus-positive stool suspensions was extracted either with the CF-cellulose method (Wilde et al., 1990) or with a simpler method using a commercial phenol-containing reagent (Maunula et al., 1999). For the CF-cellulose method, 200 µl stool suspension and 400 µl extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, 1% SDS, pH 9.5) were combined and vortexed. The mixture was extracted once with phenol (600 µl) and once with phenol and CHIS (300 µl and 300 µl; see details in RNA extraction and gel electrophoresis). The water-phase was separated and combined with 95% ethanol (80 µl, 15% v/v) and 60 mg CF11-cellulose (Whatman Chemical Separation Ltd, England). The mixture was vortexed and incubated on a rotating mixer for 90 min at 4 °C. After

centrifugation for 1 min the precipitate was washed three times with STE-ethanol buffer (0.1 M NaCl, 0.001 M EDTA, 0.005 M Tris hydrochloride, pH 7.0, ethanol 15% v/v). The fourth wash was performed with STE buffer without ethanol (500 µl) to elute the RNA. After centrifugation for 5 min the supernatant was precipitated with ethanol as described in "RNA extraction and gel electrophoresis".

For the Tripure RNA extraction method, 100 µl stool suspension was combined with 1ml Tripure reagent (Roche) and the tubes were shaken vigorously. Two hundred microlitres of chloroform were added and the shaking was repeated. The mixture was incubated for 10 min at room temperature and centrifuged at 11 500xg for 15 min at 4 °C. Ethanol-precipitation was performed in the presence of 3 M Na-acetate, pH 6.5, with glycogen as a carrier. The precipitate was washed once with 75% ethanol before use. Regardless of the RNA extraction method, the precipitate was finally dried and suspended in 50 µl distilled water.

RT-PCR. The gene amplification procedure was essentially the same for all RNA segments. For reverse transcription, a 20-µl reaction mixture was gathered containing 50 mM Tris-HCl (pH 8.8), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM dNTPs (Pharmacia, Uppsala, Sweden), 0.5 µM primer, 1U/µl RNAse inhibitor (Promega, Madison, WI), 10 U/µl M-MLV reverse transcriptase (BRL, Gaithersburg, MD) and rotavirus RNA. The template (5 µl) and dimethyl sulphoxide (3 µl) were mixed, incubated at 97 °C for 3-5 min and on ice for 3-5 min after which 6 µl mixture was used for reverse transcription which was allowed to occur at 37 °C for 45-60 min. An aliquot of this mixture (5 µl) was added to a PCR mixture (final volume 100 µl). The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.1 mM dNTPs, 0.2 µM each primer and 2.5 U/100 µl AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ). Before amplification, the samples were pretreated at 94 °C for 10 min after which 33 amplification cycles were performed as follows: 94 °C, 1.0 min: 40-42 °C, 2.0 min; 72 °C, 2.0 min and final incubation for 7 min at 72 °C. The same conditions were used for amplification of all segments except segment 4 for which a higher annealing temperature (50 °C) was used. Separate working rooms dedicated for handling pure reagents, templates and PCR products were used to avoid contamination.

Direct DNA sequencing. For DNA sequencing the PCR products were purified with the help of avidin-coated beads according to the method of Syvänen et al. (1989). Biotinylated PCR-product (30-90 µl), 10 µl beads (IDEXX, Westbrook, ME) and 50-70 µl TENT buffer (50 mM NaCl, 40 mM Tris-HCl, 1 mM EDTA and 0.01% Tween 20, pH 7.5) were combined (total volume 100 µl) and incubated for 45 min at room temperature. After centrifugation for 1-2 min at 3200xg in an Eppendorf centrifuge the supernatant was discarded and the precipitate (beads and DNA) was gently but thoroughly suspended in 50 µl 50mM-NaOH. The DNA strands were separated in the presence of NaOH for 10-15 min at room temperature after which the bead-DNA mixture

The primers used

Segment	PCR and sequencing primers	Region
1	beg 5'-caaagagcgttcatgtcttt-3'	2371-2390
	end[a] 5'-ggtagtgttggcataaattt-3'	2710-2729
	seq 5'-gttaacaaattatctgacca-3'	2452-2471
2	beg 5'-cctcaatggcgtacaggaa-3'	13-20
	end 5'-attctctacagccatatctt-3'	591-610
	seq 5'-gctaaacgtgaaaacttacca-3'	38-59
3	beg 5'-cctggatggaaattaacatat-3'	407-427
	end 5'-atggtgtgtccaatggatcc-3'	970-989
	seq 5'-gtcaaaacgctgcaacagatg-3'	480-500
4	beg 5'-tggcttcgctcatttatagaca-3'	11-32
	end 5'-atttcggaccatttataacc-3'	884-903
	seq 5'-gatggtccttatcagcc-3'	205-221
5	beg 5'-ctagatgtagaatg/attcacg-3'	885-905
	end 5'-taggcgctactctagtg-3'	1524-1550
	seq 5'-tcacaggaaaagatatatga-3'	1122-1141
6	beg 5'-actcttaaagatgctaggga-3'	51-70
	end 5'-gctgaattaatt/aactctt-3'	726-745
	seq 5'-actatgaatggaaatgaa/t/c/gtt-3'	141-160
7	beg 5'-gat/cactatt/agattggaaat-3'	521-539
	end 5'-ttgacagtgttagcttttaac-3'	1013-1033
	seq 5'-atgaactctcttcagaatgt-3'	641-660
8	beg 5'-atagctattggtcattcaaa-3'	457-476
	end 5'-cataagcgctttctattctt-3'	1034-1053
	seq 5'-catggtaaaggtcactatag-3'	706-725
9	beg 5'-ggctttaaaagagagaatttccgtctgg-3'	1-28
	end 5´-ggtcacatcatacaattctaatctaag-3'	1036-62
	seq I 5'-gtatggtattgaatataccac-3'	51-71
	seqII 5'-ggatggccaacaggatc-3'	376-392
10	beg 5'-agttctgttccgagagagcg-3'	11-30
	end 5'-gtcacactaagaccattcc-3'	731-749
	seq 5'-cacattgagtgtaatcactt-3'	68-87
11	beg 5'-tggaaaatctattggtagga-3'	110-129
	end 5'-agtggggagctccctagtg-3'	631-649
	seq 5'-ctggcgtgtctatggattca-3'	280-299

[a] The underlined primers (beg or end) have also been used for reverse transcription. The end primers were biotinylated.

was washed twice with TENT buffer. The precipitate was suspended in 10 µl reaction buffer (40 mM Tris-HCl, 20 mM $MgCl_2$ and 50 mM NaCl, pH 7.5) with 1 µM of sequencing primer. The annealing step was allowed to proceed at 37 °C for 15-30 min. The reaction buffer mentioned above was included in the Sequenase version 2.0 DNA sequencing kit (USB, Cleveland, OH) which was used for sequencing. A label, [α-^{35}S]dATP, was used for visualization of the reaction products of this dideoxynucleotide chain termination-based method (Tabor and Richardson, 1990). The products were resolved on 6% polyacrylamide, 7 M urea thin gels in taurine buffer (89 mM Tris base, 29 mM taurine, 0.5 mM EDTA, pH 8.8).

Sequence analysis. The nucleotide sequences were analysed with the help of several computer programs. For storage and simultaneous visualization of nucleic and amino acids of a single sequence, the MacMolly–program (Analyse PPC) was used. The UWGCG program package (Devereux et al., 1984) in the UNIX system was especially useful in handling the sequences. The SeqApp program of Macintosh was found to be fast and helpful for the alignment of sequences. The PHYLIP software package (Felsenstein, 1989), especially maximum likelihood (ML), was used in the phylogenetic analyses sequences (III). Since it was found to be very slow when a great number of sequences were analysed, the fastDNAML program (Olsen et al., 1994), which is derived from the DNAML program of the PHYLIP package, was used for that purpose. Phylogenetic analyses were also performed using the CLUSTAL V software package (Higgins et al., 1991) and PILEUP program of UWGCG (IV).

Nucleotide sequence accession numbers

RNA segment	G and P serotype	Genbank accession number	
Seg 4	G1P[8]	Z80234-Z80270	III[a]
Seg 9	G1P[8]	Z80271-Z80315	III
Seg 4	G4P[8]	Z80316-Z80328	III
Seg 9	G4P[8]	Z80329-80338	III
Seg 4	G4P[6]	Z80321	III
Seg 9	G4P[6]	Z80332	III
Seg 1		AJ272451-8	IV
Seg 2		AJ287448-55	IV
Seg 3		AJ287456-64	IV
Seg 4		AJ276278-91	IV
Seg 5		AJ287465-71	IV
Seg 6		AJ275894-901	IV
Seg 7		AJ275902-9 and AJ292380	IV
Seg 8		AJ275942-9	IV
Seg 9		AJ288000-17	IV
Seg 10		AJ275950-7	IV
Seg 11		AJ272531-6	IV
WA s1 and s3		AJ292378 and AJ292379	IV

[a] Indicates the publication; only nonidentical sequences were submitted.

RESULTS AND DISCUSSION

Prevalence of human rotaviruses in the metropolitan area of Helsinki (I–IV)

The study material consisted of rotavirus-positive stool samples mainly from hospitalized children with diarrhoea collected in the metropolitan area of Helsinki, Finland, from 1976 to 1997 (Figure 6). During these 21 years nearly 30 000 stool samples were analysed with EM at the Department of Virology, and 5977 rotaviruses, 731 adenoviruses and 551 SRSVs (small round-structured viruses) were identified (Table 4). Rotaviruses were found in 112-519 samples per year, which comprised 9.6-27.8% (mean value 19.9%) of all samples. Adenoviruses were found in 10-77 samples per year. The number of SRSV findings varied greatly from year to year, 3-149 positive samples, and remained low during the 1990s. Until February 1979 all negative samples were pelleted with ultracentrifugation prior to re-examination by EM, which increased the number of SRSVs considerably. For rotaviruses and adenoviruses, however, very few additional findings resulted from the ultracentrifugation. Since the diagnostic relevance of the SRSV findings was unclear at that time, the ultracentrifugation step was omitted. The advantage of EM detection is that it is a catch-all method, e.g. all groups of rotaviruses can be found. The detection limit of EM, however, is relatively low (about 10^6 virus particles per ml), and more rotavirus cases have been found by using more sensitive methods, e.g. RT-PCR (Pang et al., 1999).

The seasonal pattern typical for rotaviruses in temperate climates (review in Bishop, 1994) was seen in Helsinki district as well as in the whole country (Vesikari et al., 1999). The epidemic season usually started in November-January and ended in May-June. During summer and autumn only a few rotavirus cases were found. The reasons for this seasonality are still unknown, as they are with respiratory infections. The lower indoor humidity and indoor crowding in the colder months have been proposed as possible factors that would help rotaviruses to spread better in the environment (Brandt et al., 1982). In tropical climates rotavirus infections often appear throughout the year with no marked peak months (review in Bishop, 1994). In our study adenoviruses were found throughout the year, although in October-March they were slightly more common than in other months. The SRSV findings were also concentrated in the winter months. In the case of rotaviruses it was found to be more useful to compare epidemic seasons rather than years, which is reflected in the following results. Longitudinal analysis of molecular epidemiology of rotaviruses has also been performed in Melbourne, Australia, from 1973 to 1989 (Bishop et al., 1991).

Only a small selection of rotavirus-positive EM samples from 1976 to 1980 was stored, but since then all rotavirus-positive samples have been available for research. The

Table 4. *Viruses found in stool samples of children with diarrhoea by EM during 1976-1997 at the Department of Virology, University of Helsinki.*

Year	Rotavirus-positive (%)		Adenovirus-positive (%)		SRSV-positive (%)		Negative (%)		Total
1976	178	(27.8)	17	(2.7)	42	(6.6)	403	(63.0)	640
1977	189	(25.6)	21	(2.8)	46	(6.2)	481	(65.3)	737
1978	406	(27.3)	77	(5.2)	149	(10.0)	855	(57.4)	1487
1979	250	(21.2)	27	(2.3)	26	(2.2)	875	(74.3)	1178
1980	419	(24.5)	33	(1.9)	26	(1.5)	1230	(72.0)	1708
1981	411	(21.8)	30	(1.6)	8	(0.4)	1436	(76.2)	1885
1982	442	(19.1)	26	(1.1)	26	(1.1)	1816	(78.6)	2310
1983	327	(23.2)	51	(3.6)	10	(0.7)	1021	(72.5)	1409
1984	280	(17.8)	63	(4.0)	8	(0.5)	1219	(77.6)	1570
1985	519	(19.4)	65	(2.4)	45	(1.7)	2045	(76.5)	2674
1986	473	(18.9)	55	(2.2)	34	(1.4)	1942	(77.6)	2504
1987	176	(12.4)	20	(1.4)	26	(1.8)	1199	(84.4)	1421
1988	112	(9.6)	30	(2.6)	44	(3.8)	983	(84.1)	1169
1989	177	(14.6)	21	(1.7)	11	(0.9)	1000	(82.7)	1209
1990	184	(14.0)	36	(2.7)	9	(0.7)	1083	(82.5)	1312
1991	285	(23.6)	31	(2.6)	3	(0.3)	888	(73.6)	1207
1992	205	(19.2)	21	(2.0)	6	(0.6)	835	(78.3)	1067
1993	221	(22.8)	10	(1.0)	3	(0.3)	737	(75.9)	971
1994	203	(21.2)	29	(3.0)	3	(0.3)	721	(75.4)	956
1995	247	(23.9)	14	(1.4)	3	(0.3)	770	(74.5)	1034
1996	169	(16.8)	41	(4.0)	11	(1.1)	785	(78.0)	1006
1997[a]	104	(20.3)	13	(2.5)	12	(2.3)	384	(74.9)	513
Total	5977	(19.9)	731	(2.4)	551	(1.8)	22708	(75.8)	29967

[a] only 7 months

method used for analysis was primarily PAGE of the segmented genome of rotaviruses combined with sensitive silver staining (Herring et al., 1982). The viral RNA was extracted directly from the stool suspension before running in gel. The method has been used throughout the world in molecular epidemiologic analysis of rotaviruses (review in Bishop, 1994)). With this method a large number of samples could be run relatively easily, rapidly and at rather low cost. Before this thesis work was initiated limited electropherotype (e-type) analysis had been performed on samples during 1981, 1982, 1985 and 1986 (H. Fritze and C.-H. von Bonsdorff, unpublished). These e-types have been included here.

Figure 6. *Epidemiology of rotaviruses expressed as number of positive patient samples per month found during the observation period of 1976-1997.*

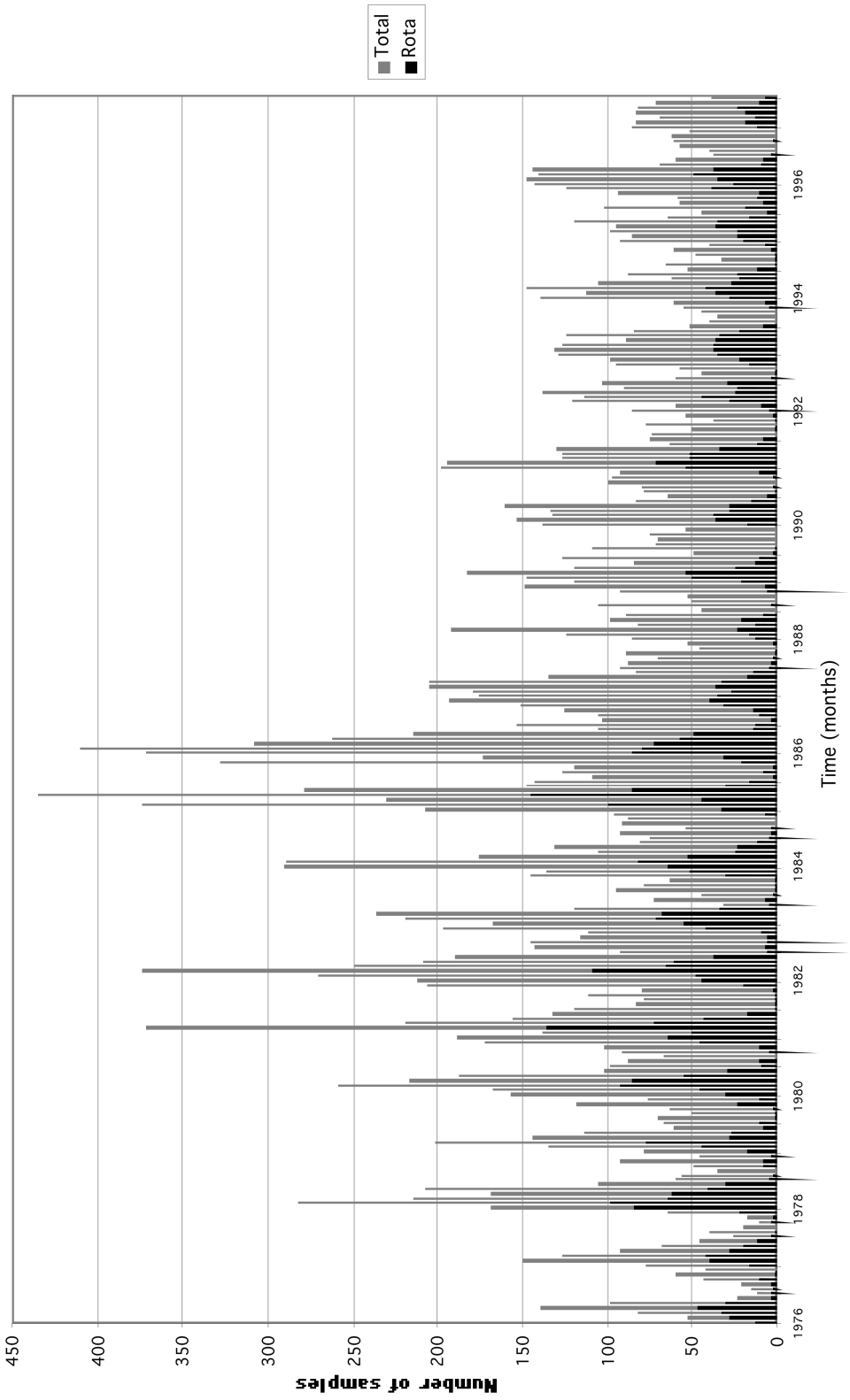

Total
Rota
Number of samples
0
50
100
150
200
250
300
350
400
450
1976
1978
1980
1982
1984
1986
1988
1990
1992
1994
1996
Time (months)

Table 5. *Gel electrophoresis analysis of rotavirus-positive stool samples originating from epidemic season 1981 to 1997.*

Epidemic Season	Group A rotavirus Short e-type	Long e-type	Group C rotavirus	Mixed e-types	Negative	No. of samples run in gel
1981	7	126	0	0	17	150
1982	2	122	1	5	8	138
1983	33	27	0	1	14	75
1984	16	192	0	3	27	238
1985	1	125	0	2	9	137
1986	0	180	3	1	17	201
1987	0	257	2	1	13	273
1988	10	80	1	5	7	103
1989	1	180	0	1	9	191
1990	2	179	5	6	10	202
1991	2	281	1	3	11	298
1992	9	154	0	3	5	171
1993	0	245	1	0	3	249
1994	0	189	6	5	3	203
1995	3	134	0	11[a]	11	159
1996	12	149	0	10	8	179
1997[b]	5	112	0	1	3	121
Total	103	2732	20	58	175	3088

[a] includes 1 rearrangement
[b] only 7 months

Group A and group C rotaviruses could be distinguished in PAGE, since the RNA pattern of group A in gel shows a 4-2-3-2-clustering of the RNA segments, while that of group C shows a 4-3-2-2- clustering. Group C was much more rarely found than group A (20 versus 2828 samples); during 9 out of 17 epidemic seasons no group C rotaviruses were found (Table 5). Most of the group A rotaviruses showed a long e-type in 2725 samples (88%); only 103 samples (3.3%) showed a short e-type. Sometimes two e-types or more than 11 RNA segments were seen in gel. In most molecular epidemiologic studies of rotaviruses analysed with e-typing, serotyping or nucleotide sequencing, samples with mixed rotavirus types have been detected. The amount of dual infections in different reports have ranged from 0.9% to 23% (e.g. Fernandez et al., 1991; Cunliffe et al., 1999; Unicomb et al., 1999; Bon et al., 2000). In our study less than 2% of samples contained more than 11 bands in gel electrophoresis. In our hands RNA electrophoresis was less sensitive than EM, since 175 out of 3088 (5.7%) samples positive with EM remained negative in gel. This result did not quite agree with those of others, who have found PAGE to be either comparable in sensitivity (Herring et al.,

1982) or even a slightly more sensitive method (Dolan et al., 1985) than EM. One reason for this may be that the samples were especially prepared for EM examination and the rest of were stored for PAGE analysis. Thus, an adequate amount of the oldest samples was no longer left for RNA extraction.

Group C rotaviruses (I, unpublished data)

During the study period 20 samples containing group C rotaviruses were detected (Table 6). The finding of the first group C rotaviruses (patients 2C-5C) in Finland was reported by von Bonsdorff and Svensson (1988). Group C rotavirus appeared to infect a different age-group than the group A rotavirus, since the median age of the group C patients was 10.3 years and that of the group A patients was e.g. 2.3 years in January 1996 and 3.2 years in August 1996. It could be speculated that more group C rotaviruses may have been detected if we had analysed adult patients. In our material about 1 out of 100 rotavirus-positive findings was a group C rotavirus. During some epidemic

Table 6. *Group C rotavirus patients encountered in the present study.*

Patient	Sex	Age (years)	Month / year of illness
1C	M	8	6 / 1982
2C	F	4	2 / 1986
3C	F	7	5 / 1986
4C	M	7	5 / 1986
5C	F	4	5 / 1987
6C	M	0.8	6 / 1987
7C	M	5	7 / 1988
8C	F	10	2 / 1990
9C	M	15	5 / 1990
10C	M	9	5 / 1990
11C	M	8	5 / 1990
12C	F	15	5 / 1990
13C	F	1.5	12 / 1990
14C	F	27	11 / 1993
15C	F	31	2 / 1994
16C	M	4	3 / 1994
17C	F	1	3 / 1994
18C[a]	F	1.5	4 / 1994
19C	F	7	4 / 1994
20C	M	41	5 / 1994
Age, median value 10.3 years			

[a] both group A and C rotaviruses were found

seasons such as in 1990 and 1994, group C rotaviruses were found more frequently than during other seasons. In 1994, a group C rotavirus case, which is not included in the study, was also found in connection with a waterborne epidemic caused by sewage contamination in Noormarkku (Kukkula et al., 1997). In most other rotavirus studies comprising mainly samples from children, group C rotaviruses have been found at about the same frequency as in our study (review in Bridger, 1987). Nilsson (2000) studied adult diarrhoea patients among whom as much as 35% of the rotavirus gastroenteritis was caused by group C rotavirus.

The RNA patterns of eight group C rotaviruses are shown in Figure 7. At least three different e-types could be differentiated. The differences appeared to occur in segments 3-4 and 8-9, which include the genes that encode for the antigenically important proteins VP4 and VP7 (segments 3 and 8, respectively). All the e-types from 1994 appeared to be similar. In this small amount of material the e-types of the group C rotaviruses did not show as much variation as was seen among group A rotaviruses, which agrees with the results obtained by others. Only a limited number of different human group C rotavirus e-types have been reported so far (Kuzuya et al., 1996). The VP4 and VP7 sequences indicate that they all belong to the same G and P serotype, although evidence for the presence of genetic clustering has been obtained (Kuzuya et al., 1996).

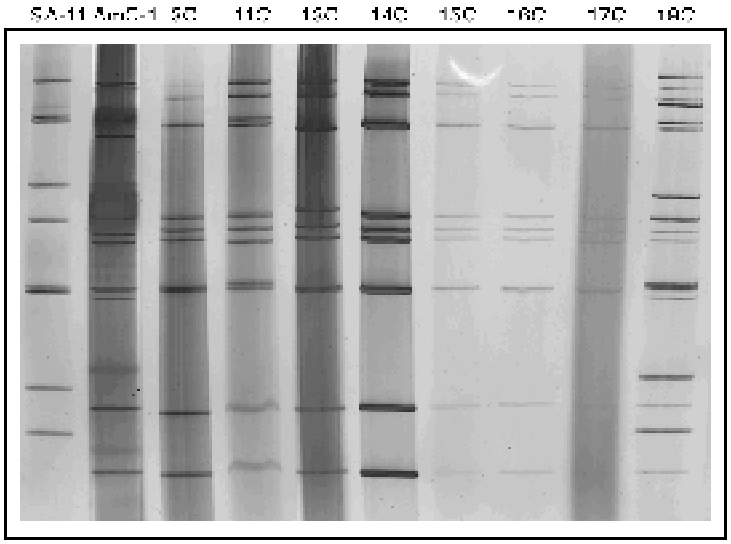


Figure 7. *Comparative gel electrophoresis of selected group C rotavirus isolates encountered in the study. Group A rotavirus SA-11 and porcine group C rotavirus rotavirus AmC-1 are included. Both group A and C rotavirus RNA segments are visible in the sample of patient 18C.*

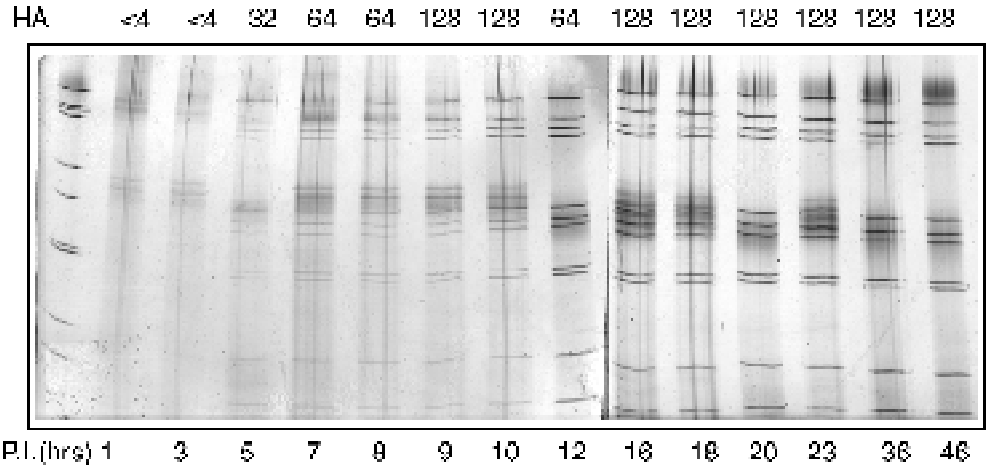


Figure 8. *Formation of viral RNA and haemagglutinin during porcine group C rotavirus infection in ST cells.*

We had an opportunity to work with a porcine group C rotavirus strain AmC-1 that had been adapted to ST cell culture by Welter et al. (1991; Figure 7). The virus and ST cells were obtained from L. Svensson in Stockholm, Sweden. The porcine virus could be grown to a titre of 10^7 pfu/ml. When the virus growth was followed in ST cells, both viral RNA and haemagglutinin (HA) were detected for the first time at 5 h p.i. (Figure 8). At 12 h p.i. a plateau was reached, after which the RNA level and HA titres remained constant. The porcine rotavirus AmC-1 was used as an antigen in the serologic tests that were set up for the analysis of anti-group C rotavirus antibodies.

We were interested in determining whether adaptation of the porcine virus to ST cells had caused amino acid changes in the VP4 protein. We sequenced segment 3 from the Sc-5 rotavirus strain, that had been passaged 6 times in gnotobiotic piglets, and that from the culture-adapted AmC-1 strain and compared the nucleotide and amino acid sequences (Table 7). Seven nucleotide differences were found, four of which changed the amino acid code. These changes were aa 108 (Asn -> Lys), aa 208 (Asp -> Asn), aa 481 (Asn -> Asp) and aa 507 (Asn -> Ser).

In 1990, a family of parents and three children suffered from a gastroenteritis episode in Lappeenranta. Rotaviruses were found in the faeces of the children, who were negative in the group A rotavirus antigen test. In PAGE, RNA patterns typical for group C rotaviruses were seen. ST cells infected with porcine group C rotavirus and fixed on glass slides were used to detect viral antibodies in patient sera with IFAT. The porcine virus antigen was also used in NT and HI assays. Group C rotavirus antibodies could be detected in the sera obtained from the father and three children,

Table 7. *Nucleotide differences between the VP4-coding gene of the published rotavirus C/Cowden strain, Cowden strain passaged in gnotobiotic pig and after cell culture adaptation.*

Nucleic acids			Amino acids		
Cowden[a]	Sc-5[b]	AmC-1[c]	Cowden	Sc-5	AmC-1
161;GC**A**	GCG	GCG	52;Ala	Ala	Ala
162;**C**GT	GGT	GGT	53;**Arg**	Gly	Gly
166;G**G**G	GCT	GCT	54;**Gly**	Ala	Ala
167;GG**G**	GCT	GCT	54;**Gly**	Ala	Ala
238;A**A**T	AGT	AGT	78;**Asn**	Ser	Ser
329;AAC	AAC	AA**A**	108;Asn	Asn	**Lys**
627;GAT	GAT	**A**AT	208;Asp	Asp	**Asn**
1447;AAT	AAT	A**G**T	481;Asn	Asn	**Asp**
1524;AAT	AAT	**G**AT	507;Asn	Asn	**Ser**
1661;GT**A**	GTG	GTG	552;Val	Val	Val
1871;GCT	GCT	GC**C**	622;Ala	Ala	Ala
1979;TTG	TTG	TT**A**	658;Leu	Leu	Leu

[a] [M74218] in Genbank

[b] 6th passage in gnotobiotic piglets

[c] cell culture-adapted and -attenuated strain

using IFAT and NT (Table 8). In IFAT, low amounts of IgM antibodies against group C rotaviruses could be detected in the children, but not in the father. Instead, the father showed a high and long-lasting IgG antibody response compared with the children in the IFAT test. His sera also expressed higher NT titres than did the children's sera. The results suggested that the children had a primary infection of group C rotavirus, but the father had a secondary infection. Two out of four sera obtained from persons that had been in contact with the family and had had diarrhoea also contained group C antibodies when tested with HI (data not shown).

In addition to this family, sera from several other group C patients and their family members suspected of suffering from the same illness were also investigated (Table 8). Antibodies against group C rotavirus were found in the sera of the four group C patients analysed (3C, 5C, 8C and 9C). Patient 9C developed a group C rotavirus infection in 1990 and a group A rotavirus infection one year later. Sera from both episodes were obtained; a significant increase in the IFAT-IgG, NT and HI titres against group C rotavirus was seen between the first paired sera. The third sera, from 1991, on the other hand, contained a high amount of group A rotavirus IgG antibodies, after which their amount started to decrease. The patient had a deficiency in IgM antibody production, and no IgM was detected in either of the virus infections.

Table 8. *Antibody detection of group C rotavirus patients by IFAT, NT and HI tests.*

Patient	Date	Group C rotavirus IFAT IgM	IgG	NT	HI	Group A rotavirus IFAT IgG
Family outbreak						
10C	I (1 wk)	16	256	20	-	-
	II (1 month)	<4	128	5	-	-
	III (5 months)	<4	64	5	-	-
11C	I (1 wk)	32	<4	20	-	-
	II (1 month)	4	256	5	-	-
	III (5 months)	<4	256	5	-	-
12C	I (1 wk)	16	256	20	-	-
	II (1 month)	8	512	20	-	-
	III (5 months)	<4	128	5	-	-
Father	I (1 wk)	<4	256	80	-	-
	II (1 month)	<4	512	80	-	-
	III (5 months)	<4	512	20	-	-
Other group C patients						
3C	I (5 days)	-	<4	40	80	-
5C	I (2 wk)	-	16	40	80	-
8C	I (3 wk))	-	-	-	80	-
9C	I (1 day)	<2	<4	5	20	<16
	II (20 days)	<2	256	256	160	<16
	III (1 year)	<2	128	32	-	1024
	IV (1.5 years)	<2	16	32	-	64

An EIA for measuring group C rotavirus antibodies in the serum samples was set up by using the porcine group C rotavirus as an antigen. A hyperimmune serum against purified virus was prepared in rabbit. An indirect EIA was used, in which the microplate wells were coated with the group C hyperimmune sera and the viral antigens were allowed to bind to the antibodies before addition of serum samples. The principle was the same in a group A rotavirus EIA, but a hyperimmune serum produced against human group A rotavirus was used as an antibody (prepared earlier in our laboratory). The serum samples were assayed for the presence of group A and group C rotaviruses by EIA (Table 9). Patient 9C showed a significant increase in the first paired sera in the group C IgG antibody levels, and a high group A IgG titre was measured in the third sample, which was well in line with the IFAT results. When more samples were analysed, it soon became evident that the increases or decreases detected in the group C rotavirus titres were often reflected in group A titres of the same patient as well. An explanation for this was sought in RIPA and immunoblotting analysis of the same sera (data not shown), but the reason for it remained unclear. In the literature there is only one report demonstrating any cross-reactivity between group A and C rotaviruses (Tsunemitsu et al., 1992b); in other reports cross-reactions have

Table 9. *IgG antibody detection in group C rotavirus patients by EIA.*

Patient	Date	Group C rotavirus EIA IgG	Group A rotavirus EIA IgG
Family outbreak			
10C	I (1 wk)	0.952	1.430
	II (1 month)	0.980	1.227
	III (5 months)	0.489	0.345
11C	I (1 wk)	0.368	0.436
	II (1 month)	0.453	0.343
	III (5 months)	0.790	0.395
12C	I (1 wk)	0.307	0.739
	II (1 month)	0.528	1.771
	III (5 months)	0.647	1.125
Father	I (1 wk)	0.994	1.029
	II (1 month)	1.521	1.120
	III (5 months)	1.360	1.081
Other group C patients			
8C	I (3 wk)	0.510	1.500
9C	I (1 day)	0.200	0.240
	II (20 days)	1.500	0.410
	III (1 year)	0.600	>2.00
	IV (1.5 years)	0.340	0.820
20C	I[a]	0.200	0.850
	II (3 wk)	0.890	1.380
	III (3 months)	0.690	1.900

[a] sample taken before illness

not been a problem. An EIA using a recombinant protein, e. g. rVP6, may be needed to set up a reliable antibody assay for group C rotaviruses.

The EIA studies with human rotavirus rVP6 protein have given seroprevalences of 43% in the UK (James et al., 1997) and 34% in South Africa (Steele and James, 1999). Indirect IF assays have been used to measure seroprevalences of 30% in the USA (Riepenhoff-Talty et al., 1997), 27% in Japan (Fujii et al., 2000) and 38% in Sweden (Nilsson et al., 2000). A family outbreak caused by group C rotavirus was also reported in the UK (Caul et al., 1990). In addition to the sporadic cases large school outbreaks in the UK (Brown et al., 1989) and Japan (Matsumoto et al., 1989) were also reported. In the former outbreak an index case vomited in the school main corridor and possibly spread the virus to the other pupils; the latter outbreak was probably of foodborne origin.

An underlying disease was thought to make patients more susceptible to group C rotavirus infection, since 3/4 patients were diabetics in the report of von Bonsdorff and Svensson (1988). This suggestion was not supported by the findings here. A more

likely explanation may be that generally diseases of adult patients with some underlying disease are carefully investigated, while healthy adults often do not even visit the doctor because of diarrhoea, and the causative agent thus remains unrecognized. A zoonotic origin of group C rotavirus infection has also been considered. No such contacts common to all were found in our cases. Domestic animals, pigs and cattle, are known to have group C rotavirus infections. The animal group C rotaviruses, however, have been shown to be different from human group C viruses. The bovine Shintoku strain belongs to a different G serotype (Tsunemitsu et al., 1996) and the porcine rotavirus Cowden most probably belongs to a different P serotype than the human group C rotaviruses (Fielding et al., 1994). Several of these infections may have originated from abroad. One of the patients developed the infection in Morocco and one had visited Spain about a week before the illness. One of the persons having contact with the family during the outbreak had also suffered from gastroenteritis symptoms for weeks after visiting Cyprus.

Distribution of group A rotavirus G serotypes (II, IV, unpubl.)

We wanted to determine which G serotypes were circulating in different epidemic seasons, and for this purpose a number of samples from each season was selected for serotyping. A commercial serotyping kit containing VP7 protein-specific mAbs against the four most common serotypes G1-G4 was used. The serotypes found in the epidemic seasons from 1976 to 1994 are shown in Table 10. Due to the limited number of samples (86) stored from the epidemic seasons 1976-1980 only a few (47 samples) were serotyped; thus the results do not show the true distribution of the serotypes. During these five seasons all four serotypes were found. Only one G1 serotype sample was detected, which is a surprisingly low number. From the epidemic seasons 1981-1994, the serotype for 12-71 samples per season was determined. In total, from 1981 to 1994 the serotype distribution was G1 62.9%, G2 5.6%, G3 3.4% and G4 28.1%. Variation in the distribution of the serotypes occurred from one epidemic season to another. Either G1 or G4 was the most frequently detected serotype per season and G1 was the only serotype that was found in every season. During season 1983 serotype G2 circulated frequently (41.7%). The short e-types, which usually represent this serotype G2, were also most frequently found in seasons 1983 and 1984 (Table 5). From then on their number has been relatively low; in many seasons none were found. The disappearance of a previously predominant short e-type in 1981 in Sweden was reported (Svensson et al., 1986).

Only 43% (412/955) of the samples could be serotyped, which is a lower value than those (from 51% to over 90%, a median value of 73.4% in six reports) reported in the literature (Nakagomi et al., 1988; Unicomb and Bishop, 1989; Gerna et al., 1990b; Bishop

Table 10. *Distribution of rotavirus serotypes during epidemic seasons 1976-1994. VP7 protein-specific monoclonal antibodies for G1-G4 were used for serotyping.*

Season	Serotype G1	(%)	G2	(%)	G3	(%)	G4	(%)	Total
1976	-		1		-		2		3
1977	-		1		-		1		2
1978	1		-		1		2		4
1979	-		5		1		10		16
1980	1		1		4		-		6
1981	19	(90.5)	2	(9.5)	-		-		21
1982	10	(52.6)	4	(21.1)	5	(26.3)	-		19
1983	6	(50.0)	5	(41.7)	-		1	(8.3)	12
1984	2	(13.3)	3	(20.0)	-		10	(66.7)	15
1985	28	(62.2)	-		-		17	(37.8)	45
1986	27	(51.9)	-		-		25	(48.1)	52
1987	70	(98.6)	-		-		1	(1.4)	71
1988	19	(79.2)	4	(16.7)	1	(4.2)	-		24
1989	11	(39.3)	1	(3.6)	2	(7.1)	14	(50.0)	28
1990	28	(51.9)	1	(1.9)	-		25	(46.3)	54
1991	2	(16.7)	3	(25.0)	1	(8.3)	6	(50.0)	12
1992	9	(69.2)	-		3	(23.1)	1	(7.7)	13
1993	5	(35.7)	-		-		9	(64.3)	14
1994	23	(71.9)	-		2a	(6.3)	7b	(21.9)	32
Total[a]	259	(62.9)	23	(5.6)	14	(3.4)	116	(28.1)	412

[a] only years 1981-94 taken into account

et al., 1991; Begue et al., 1992; Woods et al., 1992). In most studies the presence of serotypes other than G1-G4 is one likely explanation for the low serotyping values obtained. In our study most of the untypable strains during seasons 1986-1990, were sequenced and their VP7 sequences indicated that they belonged to one of the four serotypes G1-G4, as the sequencing results in the next section will reveal. Bishop and coworkers (1991) could serotype 73.2% of their samples in Australia. They noted that many of the e-types of the untypable strains appeared identical to those that were serotypable, which indicates that the results are rather due to a low sensitivity of the assay than to the presence of other serotypes in the samples. The specificity of the commercial kit we used had been compared with two other serotyping panels, and it was found to be of equal specificity to one of the panels and better than the third panel (Green et al., 1990). Rotaviruses that have lost their outer layers cannot be serotyped. Calcium has been reported to be important in stabilizing the virus particles (Fitzgerald and Browning, 1991). In our study the faecal samples were suspended in Ca^{2+}-containing buffer, but it was not added to the buffers used in the EIA. On the other hand, the older samples had been frozen and thawed several times over the

years, which may have damaged the intact virions.

In general our results agree with those reported in Australia (1973-1989; Bishop et al., 1991) and worldwide (1983-1989; Beards et al., 1989; Woods et al., 1992). Serotype G1 has predominated everywhere, but otherwise the prevalences of the serotypes have varied widely both temporally and geographically. Serotype G1 was also the most common serotype in other studies of rotavirus serotypes in Finnish children during 1984 (Vesikari et al., 1985), 1986-1987 (Vesikari, 1989) and 1993-1995 (Pang et al., 1999; Pang, 2000). Serotype G4 was the second most frequent serotype found by Pang and coworkers (1999). Gerna and coworkers (1990b) also reported serotype prevalences among European samples that included Finnish samples from Turku. The serotype distribution was G1 64.5%, G2 2.5%, G3 1.0%, G4 18.5%, mixed 1.1% and atypical 0.7%, which agree well with our results.

The epidemic season 1987 was exceptional in that sense that it began early in September. The previous epidemic season had lasted until the summer months, and the two seasons were separated only by August. We were interested in analysing the rotavirus strain that could cause such an early start in an epidemic season. Primarily for this reason the next four epidemic seasons 1986-1990 were selected for detailed analysis of the e-types of the circulating rotavirus strains.

Results from the electropherotyping analysis (II, IV)

The e-types of the four epidemic seasons 1986-1990, (named Seasons1-4) were grouped according to their similarity by comparing their RNA patterns by eye. In total, 645 out of 769 group A rotavirus e-types were clear enough to be classified into 87 different groups. Grouping of the e-types analysed in different gel electrophoresis runs proved to be difficult, since the e-type of the marker SA-11 showed slight variation from gel to gel. The human eye grouped the e-types more correctly than any scanning computer program available at that time. The disadvantages of the manual technique were that it was time-consuming, and only a limited number of e-types could be compared with each other at the same time. The grouping analysis was done step by step as follows: 1) e-types of each epidemic season were compared and grouped separately, 2) e-types (now grouped) of two successive seasons were compared (Seasons 1 and 2; Seasons 2 and 3, etc) and 3) e-types of non-successive seasons were cross-compared (Season 1 and 3 etc.), but only those e-types consisting of more than one isolate. For confirmation of the groupings and in difficult cases, coelectrophoresis of two samples in the same lane in gel was used.

The number of e-types from season to season varied from 16 to 34, so several different

e-types were circulating simultanously, as has been reported in previous studies (e.g. Rodger et al., 1981; Konno et al., 1984; Coulson, 1987). Typical characteristics included a sequential pattern of appearance of the e-types, which agreed with results in the literature (Rodger et al., 1981). Each e-type was also usually restricted to one particular epidemic season, as in other reports (Nakagomi et al., 1988), and did not reappear. Only four e-types were found in more than one epidemic season, three e-types were found in two successive seasons and only one e-type was found in nonsuccessive seasons (Seasons 2 and 4). Similar results from rarely occurring overseasonal e-types were obtained by others (Noel et al., 1991; Pipittajan et al., 1991).

Samples that had identical e-types also belonged to the same serotype. Results contradictory to our finding have been reported, one in which serotype G1 and G4 strains had undistinguishable RNA patterns (Gerna et al., 1987) and one in which the different monotypes had identical e-types (Unicomb and Bishop, 1989). On the other hand, Coulson and coworkers (1987) found that geographically and temporally limited samples that were grouped to one e-type all represented one serotype. Others have also come to the same conclusion (Nakagomi et al., 1988; Gouvea et al., 1990; Bishop et al., 1991; Pipittajan et al., 1991). The different results in this respect may have been due to different techniques in PAGE electrophoresis or in comparison of the e-types in different laboratories. A conclusion could be made that since samples of one e-type belong to the same serotype, only limited numbers of each e-type needed to be serotyped to ascertain the major serotypes in circulation (Coulson, 1987). In our study, when the serotype results were extrapolated from the e-types, the final serotype distribution was 61.2%, 2.0%, 0.5% and 29.8% for serotypes G1-G4, respectively.

In the literature one e-type has often been reported to predominate in any one season (Konno et al., 1984), and the predominant e-type usually changes from season to season (Pipittajan et al., 1991). In our study two e-types were found to be extremely frequent (100-200 isolates), one e-type in Season 1 and another in Season 3, while the other e-type groups had less than 30 isolates. The predominant e-type of Season 1, named e-type 101 (G1-87), was found throughout the season from September 1986 until August 1987, with a peak in January and a second minor peak in March 1987. E-type 101 represented serotype G1, since 44 out of 89 stool specimens gave serotype G1-positive results; the rest remained untyped. It can be deduced that as much as 97.1% of the rotavirus isolates were of serotype G1 in Season 1, since 10 of the 15 minor e-types were also of serotype G1. The serotype G1 rotaviruses were still circulating in Season 2, but now they had different e-types and no single predominant strain was found. All in all, the rotavirus epidemic appeared to be weaker in this season than in Season 1.

In Season 3 a strong rotavirus epidemic again appeared, and this time the predominant e-type 305 (G4-89) was a serotype G4 strain. This strain comprised about 80% of the isolates during the season. The first isolates were detected in late December 1988 and the last ones in August 1989; most isolates were found in January and February. In this season only about 12% of the isolates were of serotype G1. The following rotavirus epidemic (Season 4) started late, in January 1990, and as many as 34 different e-types, 14 G1 e-types and 7 G4 e-types, were identified. The G4 e-type 417 was the most common e-type in this season, although not predominant (only 14% of the isolates grouped to this e-type). E-type 417 was indistinguishable from that of the predominant e-type (305) of the previous year. As the following results will show e-type 417 proved to be e-type 305 that had reappeared after a 7-month latency.

Noel et al. (1991) reported a major shift from serotype G1 to G4 in London during the 1989-1990 season, when the predominant G4 e-type was also circulating in Helsinki. It would have been interesting to know whether e-type 305 was also circulating in London. The same e-types have been found in different cities in one country (Unicomb and Bishop, 1989), but e-types from different countries have not been compared. A similar type of shift from G1 to G4 was also reported later in 1997 and 1998 in Ireland (O'Mahony et al., 1999). It might be suggested that regular changes in the predominant serotype between G1 and G4 occur in Europe. Konno et al. (1984) found a single predominant e-type at the beginning of the epidemic season and then other e-types during the latter portion of each epidemic. In our study no such phenomenon was observed, but instead, a season with a predominant e-type was often followed by a season with many different e-types. A question arose as to whether the various e-types could be descendants of the predominant e-type.

The lack of a computer program for grouping the e-types hindered the extension of a detailed analysis of the e-types occurring throughout the entire 17-year study, since manual inspection was not feasible with such a vast amount of material. Instead, only those e-types having the highest prevalences in each epidemic season were identified (Table 11), and the predominant e-types were selected for detailed sequencing analysis. As expected, their prevalences varied widely, between 18% and 70%. The distribution of the e-types as predominant and non-predominant or short major and minor e-types was not as easy as in Seasons 1-4. Six e-types (1985, 1988, 1990, 1992, 1994 and 1995) were clearly minor ones with prevalences less than 20%, and five e-types (1981, 1984, 1987, 1989 and 1997) were clearly major ones with prevalences over 50%. Classification of the remaining e-types was problematic. We decided that those with prevalences over 30% probably also belonged to the major e-types. The serotypes of these 17 e-types were determined; ten serotype G1, six G4 strains and one G2 rotavirus strain were found. Approximately the same number of serotypes G1 and G4 were

Table 11. *Prevalences and G serotypes of the most common rotavirus e-types in epidemic seasons 1981-1997.*

Epidemic season	Prevalence (%) of the most common e-type / season	G serotype (major e-type)	(minor e-type)	Rotapositive per season
1981	50.4	G1		466
1982	47.8	G1		391
1983	44.0	G2		319
1984	62.4	G4		357
1985	17.5		G1	461
1986	39.3	G4		442
1987	74.4	G1		270
1988	13.6		G1	101
1989	69.6	G4		190
1990	12.9		G4	172
1991	45.3	G4		293
1992	6.4		G1	171
1993	31.3	G4		247
1994	17.2		G1	203
1995	11.9		G1	198
1996	25.1		G1	230
1997[a]	57.0	G1		104

[a] only 7 months

found among the major e-types (4 versus 5). In season 1983 the short e-type was predominant over the long e-types, but since the RNA patterns were not grouped further, it is not known whether a single or several short e-types were causing the epidemic.

Phylogenetic analyses (III, IV)

P genotypes and genetic analysis of the genes coding for the antigenically important proteins VP4 and VP7

The P types of the rotaviruses found during Seasons 1-4 and of the most prevalent rotavirus strains occurring throughout the observation period were determined. As the P-serotyping by mAbs has been reported to be difficult and no commercial mAbs were available, the P genotypes were determined by sequence analysis according to Gentsch (1992). A P type was deduced from the nucleotide sequence of nt 250-624 in segment 4 encoding for the spike protein VP4, a region showing wide genetic variability. The G1 and G4 e-types and about 15 e-types that had remained untypable in Seasons 1-4 were selected for the sequence analysis; the G2 and G3 strains were not included. At least one sample from each e-type group was sequenced. The results

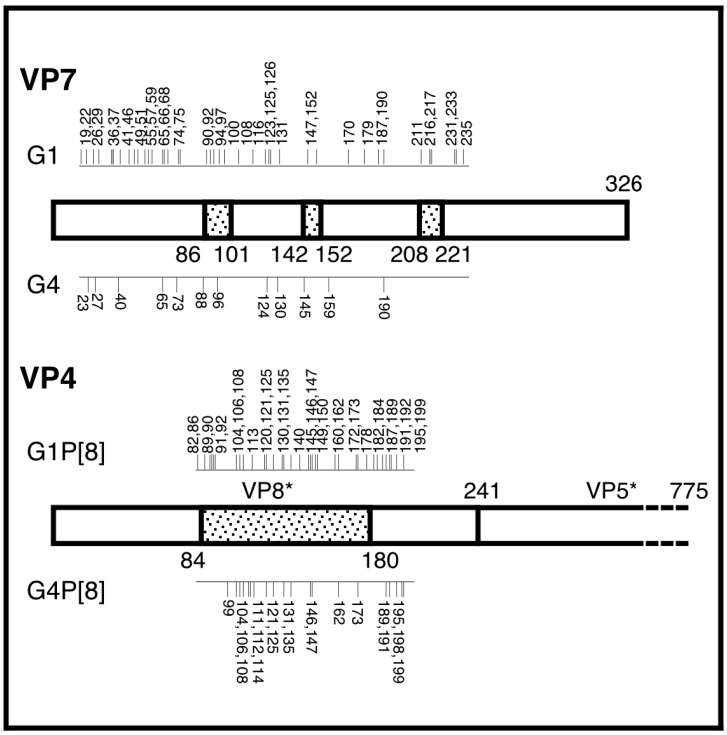


Figure 9. *Distribution of amino acid changes in 45 and 9 different sequences of G1P[8] and G4P[8] strains, respectively, in proteins VP7 (aa 18-238) and VP4 (aa 81-205). The amino acids of G1 rotaviruses are marked above both segments and those of G4 viruses underneath the segments. Positions of amino acid differences are indicated by vertical lines and position numbers. The variable regions A, B and C of segment 9 and the large variable region of segment 4 are indicated by dotted areas.*

showed that all except one e-type were of P genotype P[8], which is the most frequently found P type in combination with G1 and G4. Only one e-type had a combination of G4P[6]; this e-type (314) most probably originated from Greece.

Genetic variation was evident among strains (Figure 9), even when they had the same P genotype [8]. Phylogenetic analysis of the partial VP4 sequences clustered them into three genetic groups or lineages at nucleic acid level. One of the G1 sequences was very different from the others and formed lineage 3, while the remaining sequences were distributed into lineages 1 and 2. Nucleic acid differences of 9% and 13% were found between the lineages and 4-6% variation was found within them. Clustering did not occur so that all G1 e-types would have formed one P8 group and G4 e-types another group, but all combinations occurred. Even two e-types, one G1 and one G4, had identical segment 4 sequences. Our results agree with those of Hoshino and coworkers (1985) who found that antigenic specificities of VP4 and VP7 segregate

independently.

The P genotypes of the most prevalent e-types occurring during the 17 epidemic seasons were similarly determined; all also had a P[8] genotype. Most of the e-types, 13 strains, grouped to lineage 1 and four strains grouped to lineage 2. From those regarded as major e-types only two strains belonged to lineage 2. Genetic analyses in genotype P[8] had not been reported previously. Later, Gouvea and coworkers (1999) analysed P[8] sequences that were combined with different G serotypes (G1, G3, G5 and G9). The P[8] sequences revealed two lineages, F45-like and Wa-like that corresponded to our clusters P[8]-2 and P[8]-1, respectively. In a recent report, rotavirus P[8] sequences from the UK to three clusters as did our sequences (Iturriza-Gomara et al., 2000).

Phylogenetic analysis of segment 9 coding for VP7 protein

Partial nucleotide sequences of segment 9 of the same G1 and G4 rotavirus isolates were analysed. This genomic region (nt 100-762, aa 18-238) contained the antigenic areas A, B and C of the VP7 protein. The G1 segment 9 sequences clustered into four genetic groups; the G4 sequences were all very similar to each other and clustered together. The nucleic acid differences between the G1 groups varied between 4% and 7% and within them differences of less than 3% were seen. The VP7 lineages appeared to be more closely related than those of VP4. This may have been due only to selection of the partial sequences; a longer region including more conservative regions was sequenced in segment 9 than in segment 4. More than 50% of the sequences grouped to lineage 1 and about 19% to lineage 2, which was the most closely related group in cluster 1. A total of 13% of the sequences were of lineage 3, and one single sequence formed lineage 4. From the most prevalent G1 e-types in the 17 seasons, most segment 9 sequences belonged to lineage 1, while only two minor e-types belonged to lineage 3. This may suggest that lineage G1-1 may have had some advantage over the others; e.g. it may have been more virulent.

Xin and coworkers (1993) analysed Chinese and Japanese G1 segment 9 sequences. These sequences were divided into three subtypes: A, B and intermediate. Subtypes A and B corresponded to our clusters G1-4 and G1-3, respectively; the intermediate subtype did not perfectly correspond to any of our clusters, but included sequences belonging to cluster G1-1. Jin and coworkers (1996) analysed G1 segment 9 sequences from vaccine failures. These sequences grouped into four distinct lineages. Lineages I and II corresponded to our lineages G1-3 and G1-1, respectively. Their lineage III corresponded to our lineage 4; the lineage IV sequences were not identified among Finnish rotaviruses in this study.

Table 12. *Occurrence of G and P genotype combinations of different rotavirus e-types over four epidemic seasons.*

G and P genotype combinations	Epidemic season 1986/87	1987/88	1988/89	1989/90	Total
G1-1P[8]-1	5	9	4	11	29
G1-1P[8]-2	-	-	-	2	2
G1-2P[8]-1	4	3	1	2	10
G1-2P[8]-2	-	1	-	-	1
G1-3P[8]-1	-	-	-	1	1
G1-3P[8]-2	-	2	-	5	7
G1-4P[8]-1	-	-	-	-	-
G1-4P[8]-2	1	-	-	-	1
G4P[8]-1	-	-	2	8	10
G4P[8]-2	1	-	-	1	2

All the G4 e-types belonged to the same VP7 lineage with the G4 strains in Seasons 1-4. They were closely related to the ST3 strain, which is a prototype strain of subtype 4A (Gerna et al., 1988). Sequences related to the G4 subtype 4B (Gerna et al., 1988), a prototype of which is strain VA-70, were not found in our study. Subtype G4B was first found in a nosocomial outbreak involving newborns in Germany in 1988 (Gerna et al., 1990a), so this subtype may be restricted to infecting newborn babies. In our study the e-type 314 G4P[6] had a unique segment 9 sequence. It showed a low level of identity (about 85%) to both genotypes 4A and 4B, and thus probably represents a new G4 subtype.

G and P genotype combinations in the study material

The combinations of G and P genotypes of the rotavirus strains are shown in Table 12. The most frequent combination was G1-1P[8]-1 both in Seasons 1-4 and among the most prevalent strains in 17 years. Cluster G1-2 also preferred to combine with cluster P[8]-1 rather than with cluster P[8]-2. Instead, cluster G1-3 was clearly most commonly found in combination with cluster P[8]-2 (7 versus 1 in Season 1-4 and 2 versus 0 in 17 years). Single findings of the other G-P combinations were also detected in Seasons 1-4 with the exception of the combination G1-4P[8]-1. Even this combination was found among some randomly selected e-types during 1980-1981 and 1992. Among the prevalent strains in the longer time scale, only the two combinations G1-1P[8]-1 and G1-3P[8]-2 were found. Both the two possible G-P combinations of the serotype G4

rotavirus strains occurred, the combination G4P[8]-1 being more common, especially in Seasons 1-4.
There are to date no other reports describing the G-P genotype combinations at the level of lineages. Instead, several reports of G-P combinations at the serotype level have been published from different countries. The G1P[8] combinations have been the most prevalent: in the USA 66.4% (Santos et al., 1994; Ramachandran et al., 1998), Ireland 65% in 1995-1998 (O'Mahony et al., 1999), England 55% in 1996 (Cubitt et al., 2000) and France 1997 (Bon et al., 2000). The G4P[8] types have been the second or third most popular combination in Europe (O'Mahony et al., 1999; Bon et al., 2000; Cubitt et al., 2000) as in our study, but in the USA serotype G4 has not been as common as in Europe (Santos et al., 1994; Ramachandran et al., 1998).

E-type versus nucleotide sequences

To prove that all rotavirus isolates showing identical e-types also had identical nucleotide sequences, several isolates of the predominant e-types were analysed with partial sequencing. The segment 3 and 9 sequences of seven and eight isolates of the predominant e-types 101 and 305, respectively, were determined. Since the e-types occurred virtually throughout one epidemic season, isolates from different months were selected. The sequences of the later isolates were compared with the sequence of the first isolate within an e-type group. A total of two and five of the e-type 101 and 305 isolates, respectively, were identical to the first prototype sequence in both their segment 3 and 9 sequences. In the other isolates not more than one point mutation was found in either of the segments. From several other than predominant e-types with shorter time scales of occurrence, more than one isolate was sequenced. In most cases, they had identical segment 4 and 9 sequences within one e-type. If msny differences were detected, e.g. between the segment 9 sequences of the isolates 1 and 2 of the e-type 404, the RNA patterns of the isolates after all became slightly different, when coelectrophoresis was performed. Thus more evidence was obtained for the hypothesis that the isolates of one e-type represented the same virus strain.

We also wanted to determine whether identical e-types that circulated in different epidemic seasons still showed identical nucleotide sequences or whether point mutations had appeared. The segment 3 and 9 sequences of four isolates of e-type 417 that had an RNA pattern identical to that of e-type 305 were compared with those of e-type 305. One of the isolates had exactly identical sequences. Two isolates had one point mutation in both sequences, but were identical with each other. One isolate had three point mutations in segment 4, although the segment 9 sequence was identical to e-type 305. Thus more changes had occurred within a longer time scale, from one epidemic season to another, but still the isolates could be regarded as the same strain.

About half (6/11) of the point mutations detected resulted in an amino acid change. No accumulation of the same point mutations could be detected in the sequences, but the nucleic acid substitutions had occurred in different places along the sequence.

The lineages were frequently composed of slightly different segment 4 and 9 sequences, but often either segment 4 or segment 9 sequences were identical between different e-types. Between some e-types even both segment 4 and 9 sequences were identical. In these cases, the RNA patterns must have been different due to differential migration of segments other than 4 and 9. Interestingly, remarkably high number of e-types, most of which had appeared later than e-type 101, had identical segment 4 sequences with this predominant rotavirus. It may be speculated that either the predominant e-type 101 had changed some segment/segments other than segment 4 by reassortment with another rotavirus strain or that the other strains had changed their segment 4 with the e-type 101.

Genetic lineages at the amino acid level and the antigenic sites

The amino acid identity levels of the lineages were generally higher than the nucleic acid identity levels, as was expected. The lineages, however, still clearly existed at the amino acid level; at some particular amino acid sites, a certain amino acid residue was found in all the sequences of one lineage whereas a different residue was found in the other lineages. Although one single amino acid in one particular position was not sufficient to define a certain lineage, a combination of amino acids could do it. In VP7 G1 sequences the amino acids defining the lineages were located in the region aa 29-68. These 9-11 residues (aa 29, 37, 41, 49, 55, 57, 65-68 and possibly 74 and 75) defined the lineages of the four clusters of VP7-G1, and even three sublineages within cluster 1 could be differentiated. Why they are so conserved within the lineages is not known. Four residues (aa 29, 37, 41 and 49), are located in the signal sequence, which is cleaved off during the maturation process of VP7 in the ER. On the other hand, even without the signal sequence the remaining five to seven residues are enough to define the lineages.

Lineages 1 and 2 had no differences in their antigenic regions A, B and C. Lineage 3 was different from the 1 and 2 in regions A (aa 94; Asn -> Ser) and C (aa 217; Met -> Thr) with some exceptions. Escape mutants at both these amino acid sites have been reported (Taniguchi et al., 1988a; Coulson and Kirkwood, 1991). The single VP7 sequence of lineage 4 had one amino acid difference in region A (aa 97; Glu -> Asp) and one in region B (aa 147; Asn -> Ser). Two global rotavirus strains WA and KU belonging to this lineage also showed the same amino acid changes. Escape mutants at these amino acid sites have also been reported (Taniguchi et al., 1988a; Coulson and

Kirkwood, 1991). Diwakarla et al. (1999) proved that the phylogenetic lineages correlated with the G1 monotypes that were determined by mAbs. Lineage G1-1 reacted with monotype 1a and lineage G1-3 with monotype 1b. It remains unclear whether lineages G1-1 and G1-2 are separated as different monotypes or not. The results of Diwakarla et al. (1999) confirmed that the amino acid substitution at residue 94 (Asn -> Ser / Thr) correlated with lineage and monotype designation. This amino acid site has also been reported to be important in neutralization with other serotypes such as G2, G3, G4 and G9 (Figure 5).

The definition based on specific amino acid code was even clearer for the VP4 lineages than it was for the VP7 lineages. Four residues aa 121, 125, 131 and 135 were ISSN (Ile, Ser, Ser, Asn) or INSN (Ile, Asn, Ser, Asn) in cluster 1, VNRD (Val, Asn, Arg, Asp) in cluster 2, and INRN (Ile, Asn, Arg, Asn) in cluster 3.

Genetic analysis of all 11 RNA segments of the major e-types

The next step in resolving the puzzle of the different e-types was the determination of partial nucleotide sequences from all 11 RNA segments of selected e-types. The most dominant rotavirus strains, the nine major e-types (G1-81, -82, -87, -97 and G4-84, -86, -91 and –93) were included in the study. Regions expressing extensive genetic variation in each RNA segment were selected for sequencing after alignment of the human group A rotavirus sequences available in Genbank. Human rotavirus segment 1 and 3 sequences were so few in Genbank at that time that a region with unknown genetic variation was selected. The length of the sequenced region ranged between 246 nt and 402 nt, only in segment 1 was a shorter sequence (153 nt) determined, since the primers selected for the first time did not work with all the isolates.

Electrophoresis of the major e-types had revealed that the RNA patterns of two e-types (G1-81 and G1-82) were identical, while e-types G4-91 and G4-93 were also indistinguishable from each other. Sequencing analysis confirmed the relationship between the e-types. All 11 partial sequences of e-types G1-81 and G1-82 were identical, and the other e-type pair (G4-91 and G4-93) had only one nucleotide difference in segment 9, while the remaining sequences were identical. In contrast, all the segments of the other major e-types that showed different RNA patterns had differences also in their nucleotide sequences even to the extent that not one of their 11 gene segments sequences was identical with the others. These results give the strongest evidence so far for the hypothesis that a rotavirus e-type can be defined as a rotavirus strain.

The CLUSTAL program was used for phylogenetic analysis of the sequences. The human G1 prototype strain WA and simian SA-11 rotavirus sequences were included

in the study. Each segment was analysed separately, forming a total of 11 phylogenetic trees. The sequences in three trees showing segment 1, 3 and 10 sequences had such high levels of nucleotide sequence identity that they were regarded to form one cluster. The other eight trees expressed more genetic variation and formed two to four distinct clusters.

The gene cluster combinations of the rotavirus strains were compared. A most surprising finding was that all the major e-types with different RNA patterns also had different gene cluster combinations. This was best illustrated when the clusters within each tree were indicated by a letter, cluster A being the group containing the highest number of rotavirus sequences. For example, analysis of the three different G1 e-types showed that their segment 9 sequences belonged to cluster 9B, while the other segments belonged to cluster A of the corresponding segment, with the following exceptions. Segment 8 of e-type G1-81 belonged to cluster 8B, segment 5 of e-type G1-87-101 belonged to cluster 5B and segment 6 of e-type G1-97 belonged to cluster 6B. The three different gene cluster combinations were:

G1-81	1A2A3A4A5A6A7A8B9B10A11A
G1-87	1A2A3A4A5B6A7A8A9B10A11A
G1-97	1A2A3A4A5A6B7A8A9B10A11A

While the point mutations could have explained the few nucleotide differences within a cluster, they do not explain the fact that segment 5 belonged to cluster 5A in 1981, then to cluster 5B in 1987 and again to cluster 5A in 1997. This type of result could hardly arise from a recombination event, either. Instead, frequent reassortments among rotavirus strains could result in such a variety of genome combinations. The results also suggest that any of the 11 RNA segments can be changed between different rotavirus strains, not just those coding for antigenically important proteins.

CONCLUDING REMARKS

This study of the genetic diversity of human rotaviruses revealed that several rotavirus strains circulated at the same time during an epidemic season, even though one strain was predominant over the others during many epidemic seasons. Several serotypes and genetic lineages of one serotype were found simultaneously. The results obtained from sequencing analysis of a single segment suggested that rotaviruses genetically can be regarded to be relatively stable, unlike many other RNA viruses. This may be due to the double-stranded nature of their genome, which is unique for *Reoviridae* among animal viruses. The nucleotide sequences of this study were so short that it would not have been possible to analyse the frequency of recombination among the rotavirus strains. The significance of recombination thus remains open. The stability of the RNA sequences was, however, in contrast to the endless variety of different e-types. New rotavirus e-types emerged and old e-types died out constantly. The results of this study suggest that reassortment is the main mechanism by which rotaviruses create genetic variation. It appears that there exist relatively stable pools of rotavirus RNA segments from which the genomes of the viruses are selected. No constraints in combining the RNA segments from different pools could be detected. In recent studies in which PCR genotyping has been used for molecular epidemiologic analysis, even more samples with mixed genotypes were found (e.g. Unicomb et al., 1999) than in our study. The possibility for reassortments may be higher than has been previously expected.

Certain rotavirus e-types were predominant during one or two epidemic seasons. The reason for predominance of certain e-types is not known. The immunity of the population may have affected the shifts from G1 major e-types to G4 major e-types or vice versa that were detected during the study period. Seasons with no predominant e-types may also have arisen, because host immunity may have suppressed the major e-type. The immunity of the population did not, however, select for the major e-types over the longer time scale, since they were almost without exception serotype G1 or G4 for 17 years and only once serotype 2 and never serotype G3. It can also be speculated that mere chance makes a certain e-type predominant. Rather than superiority of the virus, other factors such as temperature and weather, the number of people a given e-type can infect at a time, and the time scale over how quickly it has an opportunity to spread at the beginning of the season, may determine when the epidemic season begins and whether an e-type becomes predominant.

Results pointing to the insignificance of immunity were obtained, since no genetic evolution in the antigenically important sites of the predominant e-types could be detected even during a long time period. No evidence of accumulation of point

mutations as is seen in the haemagglutinin of the influenza A virus was observed in the VP4 or VP7 proteins of rotaviruses. Since rotaviruses infect mainly a restricted cohort of people, children 0.5-3 years of age, every year new children with no previous immunity become available. Rotavirus strains may thus not be forced to evolve because of immunologic pressure.

In developing countries with warm climates, high population densities and low hygiene levels the serotype distribution is very different from that in Europe, the USA, Australia and Japan. While in the latter countries serotypes G1-G4 are the most common among rotaviruses, in developing countries many different G and P combinations are circulating, and new serotypes and subtypes are constantly found. In recent years, however, serotypes other than G1-G4 have also emerged in Europe. Since in our study only the major e-types were analysed during the 1990s and the possibility remains that there existed also casually circulating strains with serotype specificities other than G1-G4. Serotype G9 rotaviruses have been found in the USA, Europe and Australia since 1995 (Ramachandran et al., 1998; Bon et al., 2000; Cubitt et al., 2000; Palombo et al., 2000). Occasional findings of serotype G8 strains have also been reported. Finland was among the first countries where serotype G8P[14] was found (HAL 1166) in 1988-1986 (Gerna et al., 1990c), but in our study no serotype G8 was found. It will be interesting to see whether the long-lasting predominance of serotype G1 is replaced by other serotypes. In a study, where rotavirus samples isolated during 1997-1998 in Malawi were genotyped (Cunliffe et al., 1999), not a single serotype G1 sample was found, which is a quite unique result so far. It will also be interesting to see how rotavirus vaccination will affect the genetic diversity of rotaviruses.

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