Journal of General Virology (1997), **78**, 291–301. Printed in Great Britain

REVIEW ARTICLE

The molecular biology of caliciviruses

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Introduction

In 1995 the escape of rabbit haemorrhagic disease virus (RHDV) from experiments conducted on Wardang Island off the coast of South Australia served to focus international media attention on a little characterized but important group of animal and human pathogens (Kaiser, 1995; Holden, 1995; Rudzki, 1996; Tribe, 1996). The virus responsible was a calicivirus which first emerged in China (Liu *et al*., 1984) from where it spread rapidly across the globe. It was the virulence of RHDV that attracted Australian scientists to investigate its potential as a biological control agent for wild rabbits.

During the 1990s significant advances were made towards the understanding of the molecular biology of both human and animal caliciviruses. Caliciviruses are now established as the causative agents of a number of human and veterinary diseases. The prototypic calicivirus is vesicular exanthema of swine virus (VESV) (Cubitt *et al*., 1995) which causes vesicular lesions similar to those of foot-and-mouth disease. VESV originates from San Miguel sea lion virus (SMSV), a viral infection of marine mammals. The natural history of these viruses and their spread from marine mammals to pigs in the USA has been extensively reviewed (Bankowski, 1981; Smith, 1981; Barlough *et al*., 1986*a*, *b*). Molecular analysis has shown that VESV/SMSV isolates constitute a single genetic group within the *Caliciviridae* (Neill *et al*., 1995). In the UK, VESV/SMSV infection is considered 'exotic' and only two cases of this infection in marine mammals have been reported (Stack *et al*., 1993). Both cases occurred in seal pups in Cornwall and were mixed infections with a parapox virus. There was no clear evidence that these caliciviruses were the cause of the lesions nor were there any sequence data to confirm that the caliciviruses belonged to the SMSV group.

Other caliciviruses for which molecular data are available include RHDV, feline calicivirus (FCV), European brown hare syndrome virus (EBHSV) and the human enteric small round structured viruses (SRSVs) and 'classical' human caliciviruses (HuCVs). Details of these viruses and the diseases they cause are summarized in Table 1. Recent advances include the determination of the complete nucleotide sequences for FCV, RHDV, EBHSV, SRSVs and HuCV. In addition a new non-pathogenic rabbit calicivirus closely related to RHDV but which replicates in the gut has been described (Capucci *et al*., 1996, 1997). Significant progress has also been made towards the development of biological systems to study the replication of these viruses. A comprehensive background covering early studies on the animal caliciviruses can be found in the detailed reviews by Studdert (1978) and Schaffer (1979). The purpose of this review is to bring the new molecular data together and summarize the current status of calicivirus molecular biology.

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Morphology

The caliciviruses take their name from the characteristic cup-shaped depressions on the virions when viewed by negative stain electron microscopy (Calyx; Latin for cup or chalice). However, this must now be considered a generalization as there are caliciviruses which have an ill-defined surface morphology and a fuzzy edge to the virion as shown in Fig 1. Cryoelectron microscopy and three-dimensional image reconstruction using a primate (pygmy chimpanzee) calicivirus from the VESV/SMSV genetic group have shown that these virus particles are 40·5 nm in diameter with 32 cup-shaped surface structures comprising 90 arch-like capsomeres arranged in T = 3 icosahedral symmetry. The individual capsomeres are dimers of the viral capsid protein and show three main domains consisting of an upper bi-lobed structure, a central stem and a lower shell (Prasad *et al*., 1994*a*).

Genome structure

Members of the *Caliciviridae* possess a 7·5 kb RNA genome with a characteristic arrangement of ORFs which clearly differentiates them from members of the *Picornaviridae*. Calicivirus genomes are composed of single-stranded poly-adenylated RNA of positive polarity. A distinguishing feature of calicivirus genome organization is a 5′ non-structural polyprotein preceding the single viral structural capsid protein. A small ORF encoding a basic protein of unknown function is located at the 3′ terminus of the genome.

Sequence analyses have revealed two fundamentally different arrangements of the ORFs on the calicivirus genome.

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Table 1. Properties of caliciviruses

Virus	Morphology	Cell culture	Clinical and epidemiological features
VESV/SMSV	VESV is the prototypical calicivirus strain. VESV and SMSV are morphologically indistinguishable and display classic calicivirus morphology	Yes	Vesicular lesions similar to foot-and-mouth disease in swine. In marine mammals SMSV causes abortion and lesions on flippers
FCV	Morphology of strains is variable. Some display classic calicivirus structures others have a more fuzzy appearance like SRSVs	Yes	Severe respiratory illness, pneumonia and also limping disease in cats. Commercially available vaccine is prepared from killed virus
SRSVs	Viruses have a feathery, ragged outline. No distinctive surface structure (see Fig. 1*a*)	No	SRSVs cause epidemic nausea, vomiting and diarrhoea commonly known as 'Winter vomiting disease' in humans. Affects all age groups
RHDV and EBHSV	Classic calicivirus structures as well as more indistinct morphological forms of the virus exist in purified virus preparations	No	Fatal epidemic haemorrhagic liver disease of rabbits and the European brown hare. There is no cross-protection from prior infection by RHDV or EBHSV. A non-pathogenic rabbit calicivirus has recently been described that replicates in the rabbit gut. Prior infection with the non-pathogenic virus protects rabbits from RHDV
HuCV	Distinctive cup-shaped surface depressions giving classic 'Star of David' morphology (see Fig. 1*b*)	No	Predominantly a paediatric illness. Severe diarrhoeal illness requiring hospitalization is rare

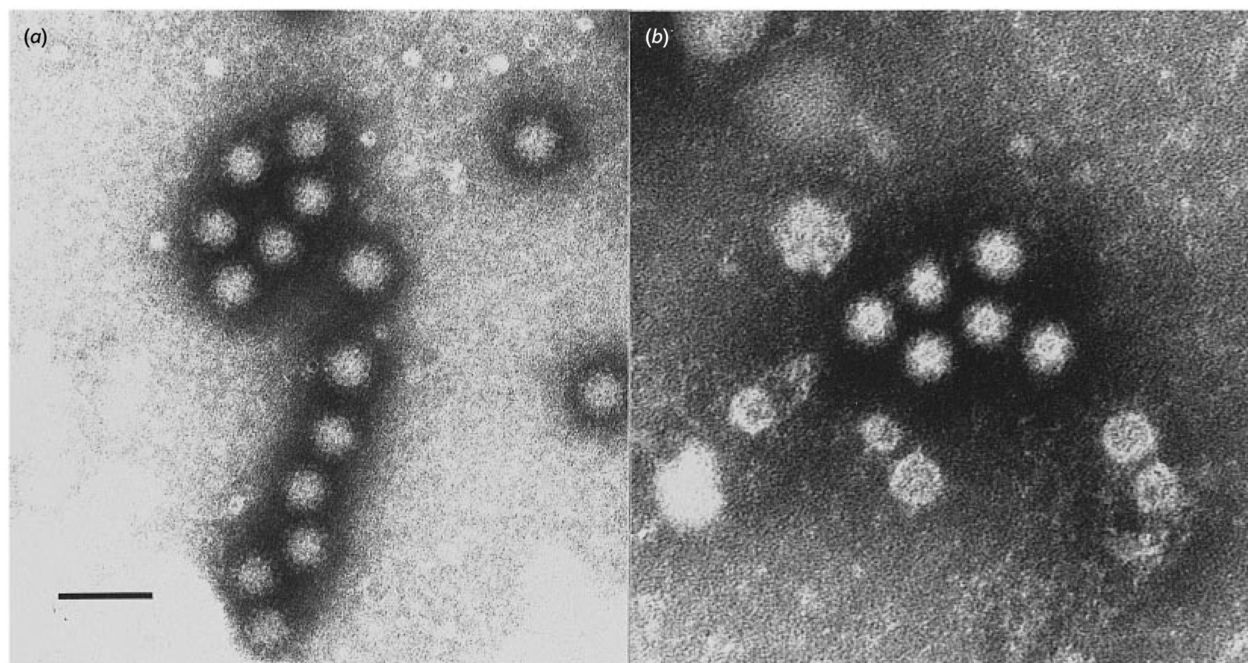

Fig. 1. Electron micrographs showing small round structured viruses (*a*) and 'classic' human caliciviruses (*b*). Viruses were negatively stained with 1·5% potassium phosphotungstic acid pH 6·5. The HuCVs shown in (*b*) are oriented along their 3-fold axis of symmetry and show the classic 'Star of David' morphology. The SRSVs were isolated from an elderly patient during an outbreak of gastroenteritis at a nursing home . The HuCV is from a sporadic case of diarrhoeal disease in an infant. Scale bar for both panels represents 50 nm.

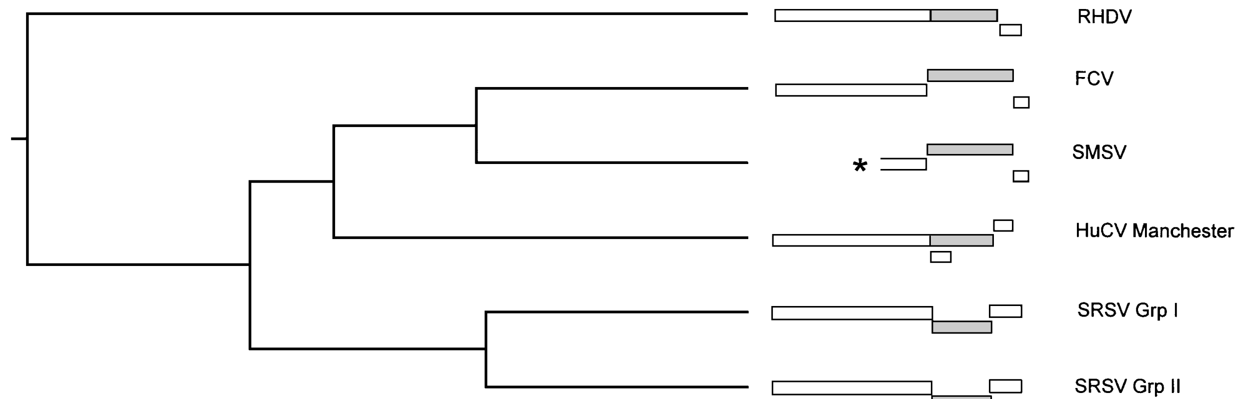

Fig. 2. Phylogenetic relationship of the human and animal caliciviruses. Sequence analyses were based on the C-terminal half of the viral RNA-dependent RNA polymerase and the lengths of the branches are proportional to the genetic relationship of the viruses. Genome organization and reading frame usage is shown by the boxes at the end of the branches. The shaded box represents the capsid structural gene and the asterisk (*) shows the limit of known sequence for SMSV.

These are exemplified by the genomes of FCV and RHDV shown in Fig. 2 where the basic difference between the two genome organizations centres around the reading frame usage at the polymerase–capsid boundary.

Separate reading frames for the capsid and non-structural polyprotein are a characteristic feature of FCV. Complete genome sequences are available for four different FCV isolates (Carter *et al*., 1992*a*; Neill *et al*., 1991; Neill, 1990; Tohya *et al*., 1991; Oshikamo *et al*., 1994; Sosnovtsev & Green, 1995) and all show a high level of similarity with a size variation of only nine nucleotides (7681–7690). The genome encodes three ORFs and in each case the first potential initiation codon for ORF1 is located at nucleotide 20 encoding a long polyprotein of 1763 amino acids. The nucleotide sequence at the junction between the polyprotein termination codon and the ORF2 initiation codon is absolutely conserved. The terminator codon for ORF1 is followed by two bases and the first base of the initiation codon for ORF2 does not overlap but is frameshifted −1 relative to ORF1 (GTT **TGA** GC *ATG* TGC). The small 3′-terminal ORF (ORF3) is similarly frameshifted (−1) relative to ORF2 (making it +1 relative to ORF1) but in this case the termination codon of ORF2 overlaps the start codon of ORF3 by four nucleotides (AAG TT*A* **TG** **A**AT). The detailed genomic arrangement of FCV is shown in Fig. 3.

Although no complete genome sequence is available for the VESV/SMSV group of viruses, partial sequences for two SMSV serotypes indicate a genome organization similar to FCV (Neill, 1992). The four nucleotides of the capsid (ORF2)–ORF3 junction are identical to those in FCV although there are five rather than two untranslated nucleotides between the stop codon of the RNA polymerase (ORF1) and the initiation codon of the capsid gene.

The arrangement of ORFs on the genome of SRSVs is very similar to that of FCV with three distinct ORFs. Extensive sequence comparison of isolates has indicated that the SRSVs can be subdivided into two genetic groups (Lambden & Clarke, 1994). At present, complete genome sequences are available for only two group I isolates [Norwalk virus (Jiang *et al*., 1993; Hardy & Estes, 1996); Southampton virus (Lambden *et al*., 1993, 1995)] and one group II isolate [Lordsdale virus (Dingle *et al*., 1995)].

The Southampton virus (SV) genome at 7708 nucleotides is some 300 nucleotides longer than the FCV genome. ORF1 begins at nucleotide 5 and encodes a polyprotein of 1788 amino acids. In contrast to FCV, the SV ORF1 overlaps ORF2 by 17 nucleotides generating a +1 frame shift. An analogous situation to FCV occurs at the ORF2–ORF3 junction where the termination codon of ORF2 and the start codon of ORF3 have only a single nucleotide overlap generating a further −1 frameshift making ORF3 return to the same frame as ORF1.

The Lordsdale virus genome is 7555 nucleotides in length and like ORF1 from SV the polyprotein is predicted to start at nucleotide 5. However, this ORF is shorter and codes for a product of only 1699 amino acids. The overlap between ORFs 1 and 2 of LV is 20 nucleotides (three longer than SV) but maintains a +1 frameshift. Similarly, ORFs 2 and 3 overlap by a single nucleotide creating a −1 frameshift returning ORF3 to the same reading frame as ORF1, giving a similar overall genome organization to SV.

The arrangement of ORFs in RHDV, EBHSV and 'classic' HuCVs is different to the genome organization of FCV. In the case of RHDV and the closely related EBHSV the ORF for the non-structural proteins is fused to the capsid as a contiguous coding sequence creating a single, giant polyprotein occupying 94% of the viral genome (Fig. 3). The four complete RHDV genome sequences deposited in the sequence database are all from European isolates and share > 95% sequence identity [EMBL/GenBank accession nos M67473 (German), Z29514 (French), Z49271 (Spanish), X87607 (Italian)]. Each sequence is exactly 7437 nucleotides in length and has a predicted

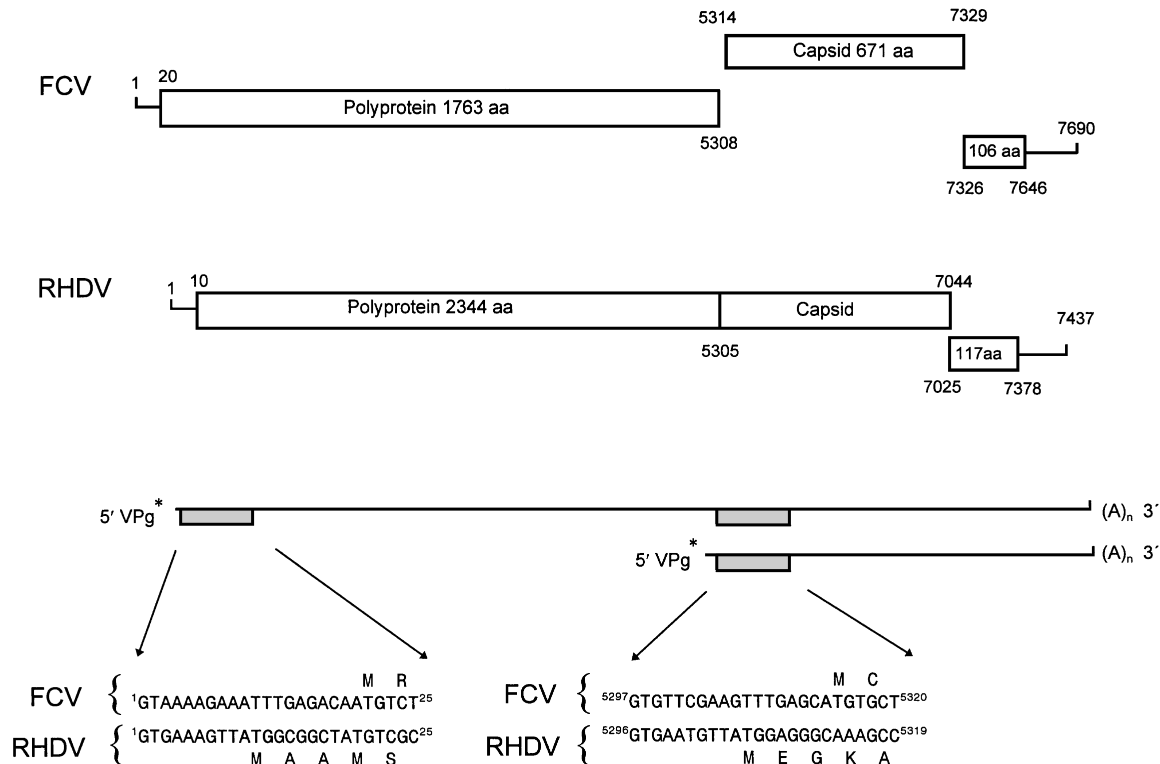

Fig. 3. Diagrammatic representation of the two different genome organisations in caliciviruses. The FCV genome (strain F9, GenBank accession no. M86379) has three potential ORFs whereas in RHDV (RHDVg, GenBank accession no. M67473) the non-structural protein ORF is fused to and contiguous with the capsid encoding region generating a single large polypeptide. Both genomes contain repeated motifs (solid block) that define the 5′ termini of the genomic and subgenomic RNAs. The 5′ terminus of RHDV genomic and subgenomic RNA is covalently linked to a small genome protein (VPg). * The presence of VPg has not been proven in FCV.

translational start codon for the polyprotein (ORF1) at nucleotide 10. ORF1 runs to position 7041 giving a product of 2344 amino acids coding for a polyprotein of predicted molecular mass of 257 kDa.

The start codon for RHDV ORF2, which corresponds by size and genome location to the small 3′-terminal ORF3 of FCV, is located at nucleotide 7025. This, in contrast to FCV ORF3, has a 17 nucleotide overlap with ORF1 but is nevertheless frameshifted −1 relative to the capsid ORF. RHDV ORF2 is 117 amino acids long and shares only 29% amino acid sequence identity with FCV ORF3. Sequence analysis of the EBHSV genome (7442 bp; GenBank accession no. Z69620) revealed a similar genome organization to RHDV (Wirblich *et al*., 1994; Le Gall *et al*., 1996). RHD and EBHS viruses share 75% amino acid sequence identity in the capsid.

Classic HuCVs are primarily associated with paediatric gastroenteritis and bear the distinctive cup-shaped surface morphology of typical caliciviruses. This feature allows HuCVs to be clearly distinguished from the amorphous SRSVs by electron microscopy (Fig. 1). A single HuCV genome sequence is available (7266 nucleotides – Manchester virus) (Liu *et al*., 1995). Studies in our laboratory and preliminary data from others (D. O. Matson, personal communication) suggest the existence of genetic variants of HuCVs analogous to the situation with the SRSVs. In the HuCV Manchester genome the region encoding the capsid protein is in the same frame and contiguous with the non-structural proteins forming one large polyprotein of 2208 amino acids. This genome organization is typical of RHDV and EBHSV; however, the characteristic repeated sequence motifs at the genome 5′ termini and at the start of the capsid gene were not found in HuCV (Liu *et al*., 1995). Although HuCV has a small 3′-terminal ORF in common with the other caliciviruses, computer analyses predict an additional ORF of 161 amino acids in a different frame overlapping with the N terminus of the capsid (Fig. 2). Although this arrangement is analogous to the candidate calicivirus hepatitis E virus (HEV), which encodes a small protein from a separate overlapping reading frame at the polymerase–capsid junction (Bradley, 1992), it is not known if the HuCV protein is functional *in vivo*. However, although possessing a positive-sense RNA genome of 7·5 kb and some morphological features of the *Caliciviridae*, HEV remains a candidate calicivirus. HEV has been reviewed extensively (Purcell, 1996) and thus will not be discussed here. The genome arrangements of VESV/SMSV, FCV, SRSVs, RHDV and HuCV are summarized in Fig. 2. Phylogenetic analysis based

on the RNA polymerase supports the subdivision of caliciviruses into distinctive 'groups' based on their biological and clinical properties.

RNA

Early work on caliciviruses adapted to cell culture (SMSV and VESV) indicated the presence of several different RNA molecules expressed in infected cells. These RNA molecules included a large single-stranded species presumed to be the genomic RNA together with smaller abundant single-stranded subgenomic RNA species (Ehresmann & Schaffer, 1977, 1979; Black *et al.*, 1978). Double-stranded RNAs resistant to ribonuclease were also described. The single-stranded molecules were polyadenylated but did not have 5′-terminal cap structures characteristic of eukaryotic mRNAs (Ehresmann & Schaffer, 1979). Further analysis of FCV-infected cells by Northern blotting with cDNA probes gave some conflicting data on the number and size of FCV-specific transcripts (Neill & Mengeling, 1988; Carter, 1990). Clearly FCV, like SMSV and VESV, also produced abundant genomic and subgenomic RNA species in addition to negative-strand copies of these RNAs (Carter, 1990). However, these experiments were unable to establish the transcriptional origin of the negative-strand subgenomic RNA. Further work is required to determine if subgenomic RNA behaves as an independent replicon. Recently, the discrepancies observed for the number of RNA species of FCV have been largely resolved (Herbert *et al.*, 1996). Polyadenylated ^{32}P-labelled RNA extracted from FCV-infected CRFK cells was separated by denaturing PAGE and only two RNA species (7·6 and 2·4 kb) were observed. Sequence analysis of the 2·4 kb RNA molecule mapped the 5′ terminus to nt 5296/7 on the viral genome as had previously been reported for the subgenomic RNA of FCV strain CF168 (Neill *et al.*, 1991). In addition, the sequence at the 5′ end of the subgenomic RNA is very similar to that at the extreme 5′ terminus of the genomic RNA. Thus, in infected cells FCV produces polyadenylated genomic RNA and a bicistronic subgenomic RNA encoding ORFs 2 and 3.

A detailed study on RHDV-infected rabbit livers also showed the presence of two abundant RNA species of 7·5 and 2·2 kb (Meyers *et al.*, 1991). Similar sized RNA molecules were observed in purified RHDV virions indicating that the subgenomic RNA was packaged into mature virus particles. The subgenomic RNA was polyadenylated and Northern blot analysis using oligonucleotides confirmed it to be of positive polarity. A similar study with a Spanish isolate of RHDV showed both genomic and subgenomic RNAs to be present in equimolar amounts (Boga *et al.*, 1992). The related EBHS virus also produces similar RNAs in infected hare livers (Wirblich *et al.*, 1994). Separation of RHDV virions by sucrose density gradient fractionation suggested the existence of two separate populations of particles carrying either genomic or subgenomic RNA (Meyers *et al.*, 1991) although it has not been unequivocally shown whether individual virions carry both RNA species. However, these workers concluded that genomic and subgenomic RNAs are separately packaged into particles. Sequence analysis of the 5′ terminus of the RHDV subgenomic RNA (Meyers *et al.*, 1991) also showed, like FCV, a conserved sequence motif of 16 nucleotides which is present at the 5′ terminus of the genomic RNA (Carter *et al.*, 1992*a*) (Fig. 3). Short internal repeated sequences located immediately upstream of ORF2 have been demonstrated in both group I and group II SRSVs (Lambden *et al.*, 1995; Dingle *et al.*, 1995). The absence of a cell culture system and the extremely low number of particles shed in these virus infections have precluded attempts to analyse the RNAs produced during infection with SRSVs. However, one preliminary report has suggested the Norwalk virus may encode a subgenomic RNA of 2·3 kb (Jiang *et al.*, 1993).

Repeated sequences at the genome and subgenomic RNA 5′ termini suggest that these structures may have a regulatory role such as providing a signal for packaging, replication or transcription of the viral genome (Lambden *et al.*, 1995). Interestingly, the candidate calicivirus HEV does not possess these repeated motifs nor are they present in the HuCV genome.

A genome-linked protein (VPg) of 10–15 kDa has been described for VESV and SMSV (Black *et al.*, 1978; Burroughs & Brown, 1978; Schaffer *et al.*, 1980). More recently, a similar protein has been identified in RHDV by radioiodination of RNA (Meyers *et al.*, 1991). In this work the presence of VPg on both genomic and subgenomic RNAs of RHDV was proven by gel separation of the iodinated RNA species. In addition, protease treatment of RHDV particles prior to phenol solubilization allowed recovery of both genomic and subgenomic RNA in the aqueous phase (Meyers *et al.*, 1991) whereas without prior protease treatment these RNAs partitioned into the organic phase suggesting the presence of a genome-linked protein. This treatment together with ribozyme digestion allowed location of the RHDV VPg to the 5′ end of the genomic and subgenomic RNAs.

The non-structural polyproteins

The linear arrangement of amino acid motifs defining the 2C helicase, 3C protease and 3D polymerase in the calicivirus non-structural polyprotein is similar to that of picornaviruses. The mature picornaviral and caliciviral non-structural proteins are released by specific viral protease activity during a proteolytic cascade (Palmenberg, 1990). The caliciviral 3C-like protease is predicted by sequence analysis to belong to a group of trypsin-like serine proteases encoded by members of the picornaviral superfamily of positive-strand RNA viruses (Dougherty & Semler, 1993). Although enteroviruses and rhinoviruses encode an additional smaller 2A protease the genomic position of the caliciviral protease suggests that the calicivirus proteases are related to the picornaviral 3C thiol

protease where the active site serine is occupied by a cysteine residue. Sequence alignments have revealed conserved motifs typical of a 3C protease located adjacent to and 5′ of the 3D RNA dependent RNA polymerase. Proteolytic cleavage of the non-structural protein precursor polypeptide has been demonstrated in RHDV (Boniotti *et al*., 1994; Wirblich *et al*., 1995; Martin Alonso *et al*., 1996) and in SV (Liu *et al*., 1996). Both viruses possess a 3C-like protease activity and the primary cleavage sites have been identified by *in vitro* mutagenesis or direct N-terminal sequencing of the proteolytic products.

The RHDV 3C-like protease has been studied in some detail and been shown to cut polyprotein substrates in *trans* and probably in *cis* (Boniotti *et al*., 1994). Proteolytic cleavage by the RHDV protease was studied both *in vivo* in *E. coli* and in an *in vitro* system using synthetic RNA transcripts translated by rabbit reticulocyte lysate (Boniotti *et al*., 1994; Wirblich *et al*., 1995; Martin Alonso *et al*., 1996). The protease expressed in *E. coli* specifically cut polyprotein substrates in *trans* and more importantly the protease was able to cleave at the junction between the capsid structural protein and the RNA polymerase. Sequence comparisons with picornaviral 3C proteases indicated four critical amino acid residues conserved in functional domains comprising a catalytic triad. These residues (H-1135, D-1152, C-1212 and H-1227) were modified by site-directed mutagenesis and the mutant protease expressed in *E. coli*. These studies clearly demonstrated that H-1135, D-1152 and the cysteine residue at position 1212 were essential components of the catalytic site and that the histidine residue at position 1227 was probably involved in substrate binding. Interestingly, substitution of the C-1212 residue with serine resulted in a mutated enzyme which retained proteolytic activity supporting the notion that this cysteine residue is the active site nucleophile characteristic of the trypsin-related proteases.

Sequence analyses of the protease region of the RHDV polyprotein suggested that this 3C-like protease was much smaller than the picornaviral 3C protease and possibly closer in size to the 2A protease (Wirblich *et al*., 1995). Expression of the C-terminal region of the viral polyprotein in *E. coli* results in release of the protease by cleavage at its N and C termini. The exact cleavage sites were determined by N-terminal sequence analysis of the cleavage products. These studies showed that the N-terminal boundary was cleaved with high efficiency at ^{1108}Glu–Gly^{1109} and that the C terminus was cleaved at a much lower efficiency between ^{1252}Glu–Thr^{1252}. The slow proteolysis of the 3CD polymerase precursor has also been reported for poliovirus where it has been proposed that this may represent an important regulatory feature because the 3CD precursor is more active in proteolytic processing whereas cleavage of 3CD is required to activate the viral polymerase (Harris *et al*., 1992). Cleavage at these two glutamic residues would yield a mature 3C-like protease consisting of 143 amino acids. Release of the polymerase from the capsid protein was also achieved by cleavage at a Glu–Gly dipeptide at position 1767. Mutagenesis studies also revealed that the cleavage specificity was primarily dependent on the amino acid residue at position 1 and that only glutamate, aspartate or glutamine were tolerated. Amino acid substitutions at position 2 had only a minimal effect on cleavage efficiency but a tyrosine residue was preferred at position −1.

Similar studies have been performed using RHDV cDNAs translated either in *E. coli* or *in vitro* using a coupled transcription-translation system (Martin Alonso *et al*., 1996). These preliminary results supported the earlier findings but additionally showed that four major translation products were produced from a full-length cDNA clone. The *in vitro* rabbit reticulocyte lysate system was expected to generate a protein of around 256 kDa representing the coding capacity of ORF1. However, four major polypeptide products with molecular masses of 80, 73, 60 and 43 kDa were observed. These polypeptides corresponded to the 2C helicase (80 kDa), a conserved domain (43 kDa), 3CD (73 kDa) and the capsid (60 kDa) regions of the polyprotein. Cleavage of the 3CD precursor was not observed but N-terminal sequence analysis of the 43 and 73 kDa fragments confirmed that cleavage occurred between Glu–Gly peptide bonds. Cleavage after a glutamine or glutamate residue is characteristic of the picornaviral 3C proteases although in contrast to the picornaviruses a glutamate residue is apparently preferred by RHDV.

More recently, a detailed characterization of the genetic map for RHDV was determined from analysis of partial and complete proteolytic cleavage products produced in a rabbit reticulocyte lysate (Wirblich *et al*., 1996). The RHDV ORF1 is processed into seven non-structural proteins plus the structural capsid protein. Proteolytic cleavage products were identified using a panel of antisera raised against recombinant peptides expressed in *E. coli* corresponding to discrete regions of the RHDV polyprotein. The location of the genome-linked VPg peptide in the polyprotein was established by immunoreactivity of radioiodinated VPg with recombinant peptide antisera. The gene order was established as NH_2-p16-p23-2C helicase-p30-VPg-3C^{pro}-3D^{pol}-capsid-COOH. The order of the viral non-structural proteins is strikingly similar to the arrangement seen in the picornaviruses and is suggestive of a common evolutionary link between the two groups of viruses. The genetic map of RHDV is summarized in Fig. 4.

A 3C-like protease activity was recently demonstrated in a human calicivirus (Liu *et al*., 1996). In the SRSV Southampton virus (SV) the protease was active in a rabbit reticulocyte system coupled to transcription of a full-length viral cDNA from a T7 RNA polymerase promoter. In this system the polyprotein translation product of ORF1 was not observed because of rapid co-translational proteolysis by the 3C protease. Three major translation products were produced and were shown, using specific antisera, to correspond to an N-terminal region (48 kDa), the 2C helicase (41 kDa) and a large 3CD precursor peptide (113 kDa). The SV 113 kDa 3CD precursor is very close in size to the combined size of the

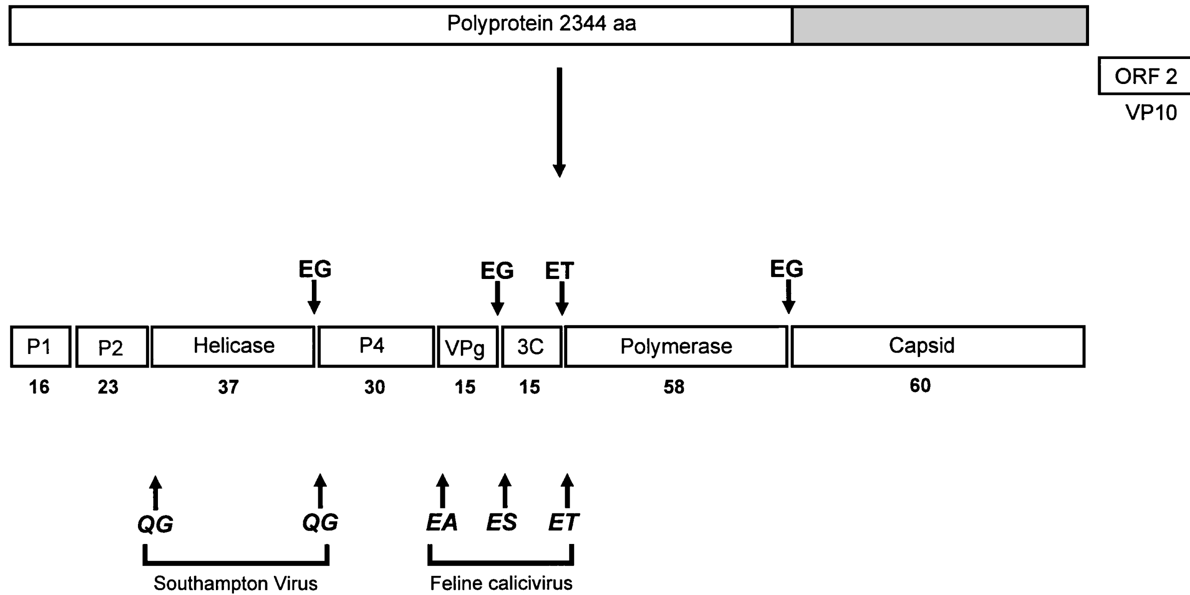

Fig. 4. Diagrammatic representation of the primary cleavage products released by 3C protease activity during polyprotein processing of the RHDV polyprotein. Translation of RNA was performed *in vitro* using rabbit reticulocyte lysate and the major cleavage products were detected by immunoprecipitation. The capsid-encoding region of the genome is represented by a shaded box and the viral protease is designated 3C. The known cleavage sites are shown as dipeptides above the protein products with the estimated molecular masses (kDa) below. The known cleavage sites for Southampton virus and feline calicivirus are shown for comparison at the bottom of the figure. The cleavage sites for FCV were kindly provided by S. Sosnovtsev.

RHDV p43 and p73 (116 kDa) and suggests that the SV protein is not cleaved at the 3C protease N terminus. The two SV proteins corresponding to the N-terminal peptide and the 2C helicase are close in size and presumably equivalent to the RHDV p80 peptide. However, the SV protein clearly undergoes a further cleavage at the putative helicase N terminus. The pattern of translation products in SV supports the model that translation initiation of the SV ORF1 occurs at one of the three tandem in-frame AUG codons at the 5′ terminus of the genome and not internally as previously thought. Studies of SV mutants generated by site-directed mutagenesis have identified the genomic location of the 3C protease and the putative active site cysteine at amino acid 1238. The exact size of the SV protease is still uncertain since complete processing to release the mature 3D polymerase was not observed. Further mutagenesis studies have identified the cleavage sites involved in the release of the SV 2C helicase between amino acid positions 399 and 762. These studies suggested that the preferred cleavage dipeptide is QG, which is also the preferred site in picornaviruses (Fig. 4). Mutagenic substitution of the critical glutamine residue with a proline residue completely abolished proteolytic cleavage. The pathways of proteolytic cleavage in RHDV and SV are clearly similar but differ in one major respect. Translation of the capsid-encoding region of the RHDV genome results in a fusion of the capsid structural protein to the RNA polymerase whereas in SV the capsid structural protein is encoded in a separate reading frame. Although the expression of the capsid protein is probably predominantly from a subgenomic message, in RHDV a further proteolytic step is required to release the mature 3D RNA polymerase from the capsid protein.

Three cleavage sites have been identified in the polyprotein of FCV (S. Sosnovtsev, personal communication). These cleavages are required to release the viral protease and VPg (Fig. 4) and as in RHDV the preferred dipeptide is occupied at position 1 by a glutamate residue.

Structural proteins

Caliciviruses possess a single major capsid protein of molecular mass 58–76 kDa (Greenberg *et al*., 1981; Madore *et al*., 1986; Terashima *et al*., 1983; Bachrach & Hess, 1973). In FCV and SMSV the capsid protein is initially synthesized as a precursor which is then cleaved by a viral protease to yield a mature protein (Fretz & Schaffer, 1978). The temporal nature of this process has been analysed in detail (Komolafe *et al*., 1980; Carter, 1989). Attempts to sequence the mature capsid protein by Edman degradation were unsuccessful suggesting that the N terminus is blocked (Neill *et al*., 1991; Tohya *et al*., 1991) However, the N terminus of the mature protein from a different FCV strain (F9) was sequenced and cleavage shown to occur between amino acid residues 124 and 125 (Carter *et al*., 1992*b*). Expression of the whole FCV capsid protein from cDNA in COS-7 cells demonstrated that the capsid precursor protein was not processed into the mature capsid (Shin *et al*., 1993) thus providing evidence that the maturation process was

not autocatalytic but dependent upon a protease expressed from the FCV genome. Preliminary antigenic analysis of FCV capsids has allowed location and identification of antibody binding sites (Guiver *et al.*, 1992; Milton *et al.*, 1992). Alignments of the capsid proteins from SMSV and FCV clearly identified six distinct regions (A–F) within the capsids (Neill, 1992). This analysis has been useful in helping to identify functional and antigenic regions of the FCV capsid (Seal & Neill, 1995; Seal, 1994; Seal *et al.*, 1993).

The first calicivirus capsid to be expressed as VLPs in insect cells using recombinant baculovirus was from the group I SRSV Norwalk virus (NV). The VLPs were exported to the cell culture supernatant which greatly aided in their purification (Jiang *et al.*, 1992). The three-dimensional structure of NV VLPs has been investigated by cryoelectronmicroscopy with three-dimensional image reconstruction (Prasad *et al.*, 1994*b*) revealing a similar structure to the primate calicivirus (Prasad *et al.*, 1994*a*). NV VLPs were immunologically indistinguishable from NV isolated from clinical samples. An immunoreactive soluble product of 32 kDa was also observed in stools containing NV. Sequence analysis of this protein fragment showed it was identical to a fragment of the rNV capsid produced by trypsin cleavage at amino acid residue 227 (Hardy *et al.*, 1995). VLPs have since been expressed for SV (group I) and the group II SRSVs Lordsdale virus (Dingle *et al.*, 1995) and Mexico virus (Jiang *et al.*, 1995). The expression of the capsid protein from these non-cultivatable viruses has provided an important set of new diagnostic reagents with which to study the seroepidemiology of calicivirus infections.

Baculovirus expression systems have been powerful tools for the production of the capsid protein from caliciviruses which cannot be grown in culture. Several groups have also expressed the RHDV capsid protein in insect cells where the protein spontaneously assembled to form virus-like particles (VLPs) which were physically and immunologically indistinguishable from the intact wild-type virions (Sibilia *et al.*, 1995; Nagesha *et al.*, 1995; Laurent *et al.*, 1994). Expression of recombinant capsid in insect cells either from an mRNA analogous to the subgenomic RNA or as part of a larger polyprotein including 3C-like protease and RNA polymerase motifs yielded VLPs indicating that capsid could be expressed by both pathways to yield VLPs (Sibilia *et al.*, 1995). VLPs contained no RNA but rabbits immunized with them were protected from challenge with wild-type virus (Sibilia *et al.*, 1995; Nagesha *et al.*, 1995; Laurent *et al.*, 1994).

3′ terminal ORF

All members of the *Caliciviridae* sequenced to date have a small 3′-terminal ORF encoding a basic protein with as yet undefined function. In FCV this ORF encodes 106 amino acids and its overall net positive charge has led to speculation that it may interact with nucleic acid and the viral capsid (Neill *et al.*, 1991). The 3′-terminal ORF is expressed from the 2·4 kb subgenomic RNA which also encodes the capsid gene (Herbert *et al.*, 1996). This protein is expressed in infected cells at approximately 10% of the amount of mature capsid. Recombinant baculoviruses expressing the RHDV capsid but lacking the 3′-terminal ORF (VP10) produce VLPs (Nagesha *et al.*, 1995; Laurent *et al.*, 1994; Sibilia *et al.*, 1995) suggesting that this ORF does not have a role in particle assembly. However, it was recently shown that the RHDV VP10 is a minor structural component of mature virions suggesting that it may play a role in encapsidation of the genomic RNA (Wirblich *et al.*, 1996).

Conclusions

The classification of SRSVs as members of the *Caliciviridae* has emphasized the importance of this family of viruses as pathogens of man and animals. However, progress on the molecular characterization of the human enteric caliciviruses has been slow because of the lack of a cell culture system and the difficulty of obtaining suitable clinical samples. Studies on the replication of caliciviruses have lagged behind the rapid advances made with the picornaviruses despite the fact that both FCV and SMSV can be propagated readily in tissue culture cells (Studdert, 1978) and that genomic RNA purified from SMSV virions is infectious (Schaffer, 1979). Recently, a full-length genomic RNA was produced *in vitro* from a recombinant cDNA of FCV (Urbana strain) (Sosnovtsev & Green, 1995) and was used to establish that a 5′-terminal cap structure is required for the RNA to be infectious. This important advance has now opened the way for genetic manipulation and biological analysis of the calicivirus genome. The development of transfection systems together with transcription-translation studies using cloned viral cDNA *in vitro* should lead to rapid progress towards understanding mechanisms involved in caliciviral replication. Until cell culture techniques are developed for the propagation of these viruses recombinant DNA technology will be important for the production of viral proteins in heterologous systems. Expression of recombinant capsid proteins as VLPs has generated material for studying the three-dimensional structure of caliciviruses by cryoelectronmicroscopy as well as providing resources for the development of viral vaccines and diagnostic reagents. Most recently, Norwalk virus VLPs have been used to investigate the attachment and entry of the recombinant capsid into cultured cells (White *et al.*, 1996). Furthermore, Norwalk virus capsids have been expressed in both transgenic tobacco and potatoes where they self-assemble to form VLPs (Mason *et al.*, 1996). Mice fed on potato tubers developed a serum IgG response to NV demonstrating the potential usefulness of plant technology in vaccine development.

In the last few years, caliciviruses have received greatly increased attention and as research interest continues to grow the future promises to bring rapid and exciting developments in the molecular biology of these important pathogens.

The authors are grateful to the Wellcome Trust (Grant no. 038076) and the Department of Health for financial support. We thank our colleagues in the Molecular Virology group and Dr E. O. Caul (Bristol Regional Virus Laboratory) for helpful discussions during the preparation of this manuscript. The authors also wish to thank Drs M. Konig and L. Capucci for the provision of data prior to publication.

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