JOURNAL OF VIROLOGY, Apr. 1996, p. 2605–2610 Vol. 70, No. 4
0022-538X/96/$04.00+0

Polyprotein Processing in Southampton Virus: Identification of 3C-Like Protease Cleavage Sites by In Vitro Mutagenesis

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Received 4 October 1995/Accepted 16 January 1996

A genomic clone of the small, round-structured virus Southampton virus (SV) was constructed from a set of overlapping PCR amplicons. Sequence analysis confirmed the absence of mutations and accurate ligation of the PCR products. The SV cDNA was cloned into a vector for in vitro production of RNA and subsequent translation by rabbit reticulocyte lysate. Two polypeptides corresponding to the N-terminal and C-terminal regions of the viral polyprotein were expressed in *Escherichia coli* and used to produce murine antisera for detection of translation products. Three major translation products of 113, 48, and 41 kDa were identified in a coupled transcription-translation system. The large 113-kDa protein reacted with antisera raised against the C-terminal region of the polyprotein and represents a precursor of the viral RNA polymerase. The 48-kDa protein detected in vitro reacted specifically with antisera raised against the polyprotein N terminus, showing that translation was initiated in SV at the three tandem in-frame AUG codons at the 5′ end of the genome. A series of nested 3′ deletions of the large open reading frame encoding the viral polyprotein was used to define the translation initiation site and genomic location of the viral protease. The results are consistent with a model in which translation of the viral genome is initiated at one of the three in-frame AUG codons starting at nucleotide position 5 and in which active viral protease is produced following translation of a region located between *Nhe*I (nucleotide 3052) and *Sph*I (nucleotide 4056), resulting in rapid cleavage of a large precursor protein. Abolition of the viral 3C-like protease activity by site-directed mutagenesis of the putative active-site cysteine (Cys-1238) resulted in production of a large protein of approximately 200 kDa which reacted with both N-terminal and C-terminal antisera. Two potential polyprotein cleavage sites containing the preferred picornaviral QG recognition site were identified on either side of the putative 2C-like helicase region of the polyprotein. Proteolysis at these positions would give rise to products with relative molecular masses identical to those of the products detected in the rabbit reticulocyte system. Site-directed mutagenesis was used to introduce a single base change which resulted in the substitution of glutamine residues with proline residues at amino acids 399 and 762. These mutations completely abolished cleavage of the polyprotein at these positions and gave rise to alternative products with molecular masses which matched the predicted sizes for a single cleavage at either Q-399 or Q-762. These data indicate that the small, round-structured virus Southampton virus produces a 3C-like protease which has two primary cleavage sites at positions 399 and 762. Proteolytic cleavage at these positions releases the putative viral 2C-like helicase.

Small, round-structured viruses (SRSVs) or Norwalk-like viruses are a major cause of epidemic acute viral gastroenteritis with outbreaks occurring sporadically in families and, more importantly, in semiclosed communities such as hospitals, nursing homes, schools, and cruise ships (15). Since the original description of the prototype agent, Norwalk virus (NV), in 1972 (16), morphologically similar viruses have been identified in numerous outbreaks of nonbacterial gastroenteritis worldwide (6). In the absence of an agreed classification system, these viruses are currently referred to by the geographic locations where they were first isolated. Until recently, the exact taxonomic status of these viruses remained uncertain and little was known about their molecular biology. However, the recent publication of the genome sequence and organization of three SRSVs, NV (14), Southampton virus (SV) (18, 20), and Lordsdale virus (7), has assigned these viruses to the *Caliciviridae* family.

The genomic organization of caliciviruses differs from that of picornaviruses, which also have a single-stranded RNA genome with positive polarity and a polyadenylated 3′ terminus (19). In picornaviruses, a single long open reading frame (ORF) encodes both the structural capsid proteins and the nonstructural proteins (28). The large picornaviral polyprotein is processed cotranslationally by a proteolytic cascade, resulting in a number of smaller structural and nonstructural proteins (8, 26). In addition, the structural capsid proteins of picornaviruses are encoded at the 5′ end of the genome, contrasting with the organization seen in two typical animal caliciviruses, feline calicivirus (5) and rabbit hemorrhagic disease virus (23). The capsid proteins of feline calicivirus and rabbit hemorrhagic disease virus are produced from an additional smaller subgenomic message which is transcribed from a region of the genome close to the initiator codon of the capsid gene (24, 25). In SRSVs, the nonstructural proteins of SV and NV are encoded by a single long ORF at the 5′ end of the genome with the capsid protein encoded in a second, overlapping reading frame, resembling the organization found in feline calicivirus (14, 18). The caliciviruses, including SV and NV, also possess a third small overlapping ORF at the 3′ end of the genome which encodes a protein with an unknown function. Partial genomic sequencing of the RNA polymerase region of several SRSVs has indicated significant genomic diversity (9) and suggested that these viruses may fall into two distinct genetic groups (2). Detailed comparative amino acid

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sequence analyses of the RNA polymerase region of ORF1, the capsid gene, and ORF3 have confirmed that SRSVs fall into two phylogenetic groups and are distinct from the animal caliciviruses (7, 10, 21, 22).

Unfortunately, the Norwalk-like viruses have remained refractory to growth in cell culture. However, the availability of genomic cDNA clones of these viruses permits the use of in vitro systems for the study of viral protein synthesis. Sequence analysis of the polyprotein of caliciviruses and SRSVs has revealed structural motifs typical of the picornaviral 2C-like helicase, 3C-like protease, and 3D RNA-dependent RNA polymerase, strongly suggesting that posttranslational proteolytic processing must also be a feature of the caliciviruses.

It has been reported that in a cell-free rabbit reticulocyte lysate system, translation of the NV polyprotein is initiated at the fifth in-frame AUG codon to produce an unprocessed polyprotein of approximately 160 kDa (14). In addition, a 5′ genomic fragment of NV consisting of nucleotides (nt) 1 to 2535 expressed by recombinant baculovirus generated an immunoreactive protein of 57 kDa in infected insect cells, supporting the hypothesis of translation initiation at the fifth AUG codon. Assuming that the closely related viruses SV and NV are likely to produce functionally similar proteins, it seemed unlikely that the fifth AUG could be the authentic initiator codon since there is no direct counterpart in SV. If the group II viruses also produce proteins functionally equivalent to the group I viruses, then the sequence diversity (7) would argue against the notion that the fifth AUG of NV is the authentic initiator codon. The additional sequence data reported for SV (20) include three tandem in-frame AUG codons which extend the frame of ORF1 by 51 amino acids to start at nt 5. This additional sequence would be expected to have a significant effect on the initiation of translation. It was therefore of interest to investigate polyprotein processing and translational controls operating in the closely related virus SV. We constructed a full-length clone of SV and found evidence that the genome of this virus is translated from the first in-frame AUG into a polyprotein that is cleaved rapidly at two distinct sites by a 3C-like protease.

Construction of a genomic clone. The virus used (Southampton/91/UK) has been described previously (18, 20) and is genetically related to but antigenically distinct from the prototype NV. Viral RNA was extracted directly from stool by using RNAzol and amplified by reverse transcription-PCR as previously described (18, 20). The complete genomic cDNA of SV was assembled from four overlapping PCR fragments, each approximately 2 kb long, and generated by using primers based on the SV sequence. The primer pairs for the four fragments were as follows: fragment 1, SV84 (5′-^{13}GGCGTCGAAAGA CGTCGTTGCAAC^{36}-3′) and SV29 (5′-^{2232}CTGTTAATGG TTGTTGGCTT^{2213}-3′); fragment 2, SV30 (5′-^{1824}TGATGCT GTGGGACGACT^{1841}-3′) and SV43 (5′-^{4269}TGCTCACCTA ACTCCCTAA^{4251}-3′); fragment 3, SV53 (5′-^{3917}CCAAATTC TGGAAATCATCC^{3936}-3′) and SV21 (5′-^{6287}TGGTTTGCC ATCCACCTC^{6270}-3′); fragment 4, SV35 (5′-^{5780}TACATTGT TTCCCCATGTGA^{5799}-3′) and SV85 (5′-^{7708}AACACTAAT CAATAGCCAAATTATTTAC^{7681}-3′). The fragments were ligated by using the unique internal restriction sites (*Kpn*I, *Sph*I, and *Xho*I), and the termini of the two outer fragments were defined by PCR primers complementary to the 5′ and 3′ termini of the SV genome. The 5′ terminus of the 5′-terminal PCR primer was synthesized by starting at position 13 to avoid problems of complementarity with the repeated sequence at the start of ORF2 (20) and because the 5′ terminus was subsequently to be digested with *Aat*II for insertion of an oligonucleotide which placed the T7 promoter sequence immediately adjacent to the extreme 5′ terminus of the SV genome. The individual PCR fragments were cloned into vector pSP73 and subjected to full sequence analysis to ensure the absence of mutations before final assembly of pSV1 carrying the complete viral genome. After construction of the complete genome, the sequences of all cloning junctions were further checked for accurate ligation.

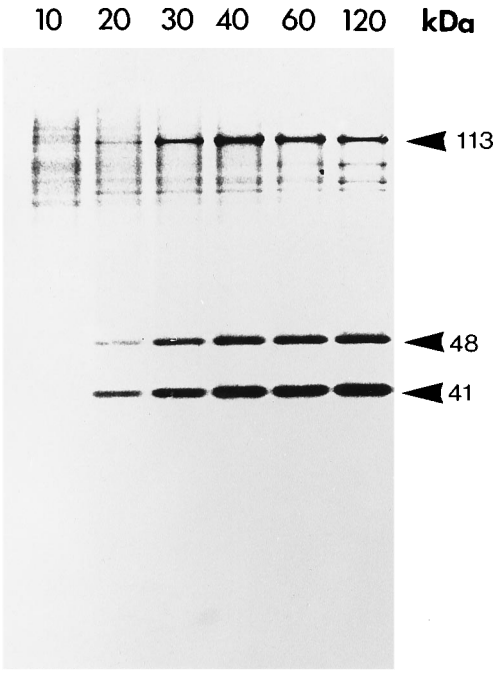

FIG. 1. Time course of in vitro transcription-translation. The TNT reactions were sampled at the times indicated above the lanes (minutes), and the L-[^{35}S]methionine-labelled reaction products were detected by SDS-PAGE and autoradiography. The molecular masses of the major translation products are shown on the right.

For in vitro studies, it was necessary to optimize the position of the SV sequence relative to an appropriate RNA polymerase promoter. Placement of the T7 promoter adjacent to the 5′ SV genomic terminus (underlined) was achieved by insertion of the oligonucleotide linker 5′-CGGATCCTAATACGACT CACTATAGGTGAATGATGATGGCGTCGAAAGAC GT-3′, following digestion of pSV1 with *Aat*II, which removed a fragment containing the SP6 promoter, a part of the pSP73 polylinker, and the 5′ terminus of SV. To produce polyadenylated RNA, the entire T7-SV cassette was excised by *Sac*I digestion and subcloned into vector pSP64-polyA to give construct pT7SVpolyA.

Analysis of translation products. In vitro protein synthesis was performed by using a T7 RNA polymerase-coupled reticulocyte lysate system (TNT; Promega, Southampton, United Kingdom) in accordance with the manufacturer's instructions. The reaction mixture (total volume, 25 μl) was incubated at 37°C, and samples were taken over a period of 2 h for direct analysis of reaction products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (17). Gels were stained and prepared for autoradiography by treatment with 1 M sodium salicylate–50% methanol for 30 min at room temperature. Gels were then dried under vacuum and exposed to Kodak XAR-5 film at −70°C. The results (Fig. 1) show that three major translation products with estimated molecular masses of 113, 48, and 41 kDa were visible after 20 min of incubation at 37°C. Expressed products from ORF2 and ORF3

were not detected, either directly or with specific antisera, indicating that internal initiation in the genome at these positions does not occur in vitro. It may be that expression of the capsid and the small 3′ ORF is primarily from a subgenomic RNA. A protein with a predicted molecular mass of around 200 kDa would be expected for the unprocessed polyprotein, although this product was never observed in time course experiments, suggesting cotranslational cleavage of the polyprotein precursor protein. The combined molecular mass of the three major translation products was estimated to be 202 kDa, which is in close agreement with the predicted size of a polyprotein initiated at the first AUG at nt 5. The major products increased in intensity for up to 60 min, but no further proteolytic processing was observed after 2 h. The simple pattern of the major proteolytic cleavage products suggested that it may be possible to confirm the predicted chromosomal location of the viral 3C-like protease and, in addition, identify the sites on the viral polyprotein where the primary cleavage reactions occur.

Immune analysis of cleavage products. Antisera to the N- and C-terminal regions of the SV polyprotein were raised against $(His)_6$-tagged fusion peptides expressed in *Escherichia coli* JM101 from pRSET vectors in accordance with manufacturer's (Invitrogen) instructions. For expression of the N-terminal fragment of SV, an 840-bp *Rsa*I-*Pst*I fragment encoding nt 150 to 990 of the SV genome was ligated into pRSET-C. The C-terminal fragment encoding the viral RNA polymerase was obtained by *Nco*I digestion of pT7SVpolyA to produce a 1,806-bp fragment (nt 3643 to 5449) which was subsequently ligated into pRSET-A. Expressed SV polypeptides were solubilized in denaturing guanidinium buffer and purified by affinity chromatography on ProBond Ni^{2+} resin. Polyclonal antisera to the two purified recombinant N- and C-terminal peptides were raised in male BK:W mice. Preimmune serum was obtained from each mouse prior to subcutaneous immunization on day 1 with 50 μg of recombinant protein in Freund's complete adjuvant, and booster immunizations were given on days 12, 22, and 33 with 50 μg of protein in Freund's incomplete adjuvant.

Sequence analysis had revealed the presence of a motif typical of 3C-like thiol proteases in the region of the genome between the *Nhe*I and *Sph*I restriction sites upstream of the RNA polymerase. The equivalent location in rabbit hemorrhagic disease virus has been shown to encode the 3C-like protease (3). To confirm the region of ORF1 encoding the SV protease, a nested series of C-terminal truncations were constructed in pT7SVpolyA. A synthetic oligonucleotide encoding a termination codon in each of the three reading frames was inserted at one of six unique restriction sites. Vector pT7SV polyA was digested with a series of restriction enzymes (*Pst*I, nt 990; *Hin*dIII, nt 1497; *Kpn*I, nt 2204; *Nhe*I, nt 3064; *Sph*I, nt 4068) to create a nested set of C-terminal deletions. Following digestion, the termini were repaired with T4 DNA polymerase, and a synthetic self-complementary termination adapter (5′-C TTAATTAATTACCCGGGTAATTAATTAAG-3′) carrying stop codons in all three reading frames was ligated into the cleavage site (Fig. 2A). The plasmid constructs were purified and used in the TNT coupled transcription-translation system. Radioactive translation products were analyzed directly by SDS-PAGE (Fig. 2B) in addition to analysis by a radioimmune precipitation assay using N-terminal and C-terminal antisera. Translation products from the TNT system (6 μl) were incubated with 2 μl of undiluted mouse antisera in 600 μl of radioimmune precipitation assay buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.15 mM NaCl, 0.1% SDS, 0.5% Empigen BB [*N*-dodecyl-*N*,*N*-dimethylglycine], 0.1 mM phenylmethylsulfonyl fluoride). After incubation at 37°C for 1 h, goat anti-mouse immunoglobulin G attached to beaded agarose (Sigma) was added to adsorb immune complexes. The beads were washed three times in radioimmune precipitation assay buffer and once in phosphate-buffered saline before derivatization in sample dissociating buffer and separation by SDS-PAGE. Translation products were detected by autoradiography. The results (Fig. 2C) showed that proteolytic processing of the polyprotein did not occur until the region of ORF1 marked by the *Sph*I site at nt 4068 was translated into protein, confirming that this region of the viral genome encodes the protease. The truncated translation product sizes were very close to those predicted for unprocessed proteins initiated at the first in-frame AUG at position 5. The major products of 48 and 41

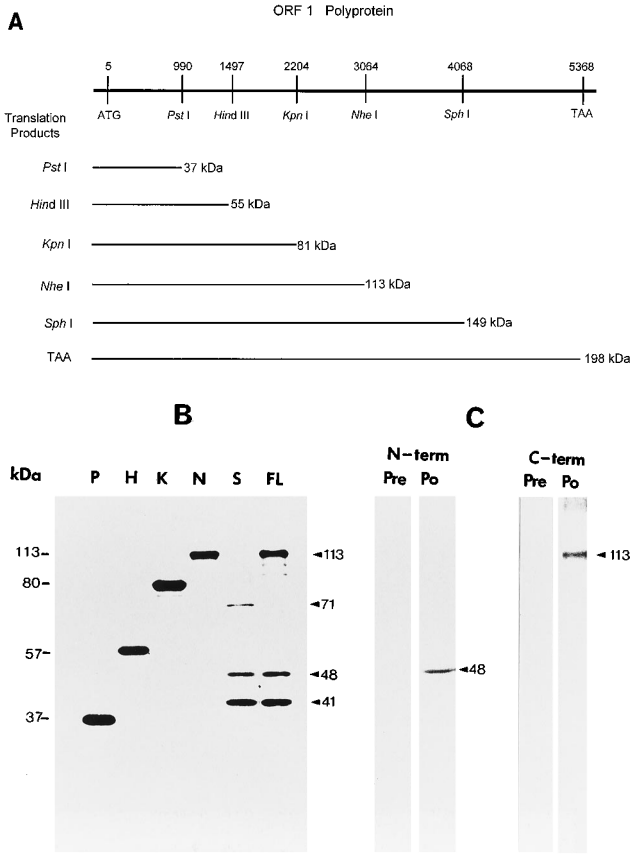

FIG. 2. (A) Diagram showing details of the series of nested 3′ deletions of SV ORF1. A synthetic adapter carrying terminator codons in all three reading frames was cloned into the restriction sites indicated. The numbers above the restriction sites refer to their nucleotide positions in the SV genome. The horizontal bars below the ORF1 polyprotein show the calculated molecular masses of the polypeptides predicted from the known amino acid sequences when translation is initiated from the first AUG at nt 5. (B) SDS-PAGE of ^{35}S-labelled products produced by in vitro transcription-translation from a nested set of 3′-terminal deletions. The observed molecular masses of the transcription products are indicated adjacent to the lanes. The restriction sites modified by the terminator adapter are shown above the corresponding translation products as follows: P, *Pst*I; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; S, *Sph*I. Lane FL contained translation products generated from a clone carrying full-length ORF1. (C) Radioimmunoprecipitation analysis of in vitro transcription-translation products produced by full-length ORF1. Translation products were captured onto a solid phase by using an anti-ORF1 N terminus (left panel) or C terminus (right panel) antiserum and analyzed by SDS-PAGE and autoradiography. Lanes Pre and Po show immunoprecipitation experiments with preimmune and immune sera, respectively. The observed molecular masses (kilodaltons) of the immunoprecipitated products are shown.

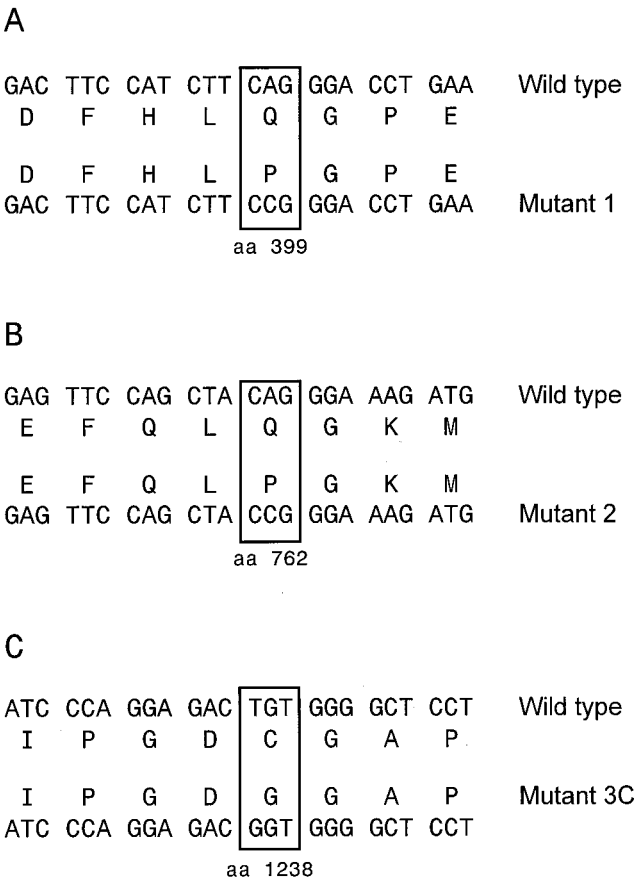

FIG. 3. Details of site-directed mutagenesis at Gln-399→Pro-399 (A), Gln-762→Pro-762 (B), and Cys-1238→Gly-1238 (C) at the active site of the 3C-like protease. Amino acids (aa) are numbered from the first in-frame methionine codon at nt 5.

kDa were produced in the *Sph*I terminal clone, showing that these proteins were not derived from the viral polymerase. Immunoanalysis of the translation products revealed that the 48-kDa protein was derived from the N terminus of the polyprotein and that the 113-kDa protein, although similar in size to the *Nhe*I N-terminal protein, is the C-terminal half of the polyprotein and is composed of at least the RNA-dependent RNA polymerase and the 3C-like protease. The 41-kDa protein is likely to be the processed 2C-like helicase, as revealed by the mutagenesis studies described below.

Mutagenesis of the 3C-like protease. The size of the uncleaved polyprotein and the possibility of additional proteases encoded by ORF1 were investigated by inactivation of the 3C-like protease. To focus on processing of the nonstructural polyprotein, a derivative clone carrying only ORF1 (pT7SVORF1) was used to generate mutant clones. By analogy with the picornaviral 3C-like protease, the conserved GDC motif in the SV genome is suggestive of a thiol protease with an active-site cysteine. Mutagenic primers were used to replace the thymine residue at nt 3716 with a guanine residue, resulting in a cysteine-to-glycine substitution at amino acid 1238 in the polyprotein (Fig. 3C). The point mutation T-3716→G-3716 (Cys→Gly) was introduced with mutagenic primers by using overlap extension PCR (12, 13). Briefly, the target region of the genome, flanked by unique restriction sites, was amplified in two sections by using complementary forward and reverse mutagenic primers. The complete fragment was generated by annealing of the two primary PCR products, followed by further rounds of PCR with a nested set of flanking primers. The primary PCR products for the T-3716→G-3716 mutation were generated with the following primer pairs: SV51 (5′-^{2807}GT GGTAGAAAAGTATGGGC^{2825}-3′) with SVmut2 (5′-^{3727} AGGAGCCCCAC**C**GTCTCCTGGGAT^{3704}-3′) and SVmut1 (5′-^{3704}ATCCCAGGAGAC**G**GTGGGGCTCCT^{3727}-3′) with SV43 (5′-^{4269}TGCTCACCTAACTCCCTAA^{4251}-3′). The complete fragment was generated by annealing of the two primary PCR products, followed by further rounds of PCR with nested primers SV73 (5′-^{3008}GGTGGCAATTATAGCATACA^{3027}-3′) and SV105 (5′-^{4122}AATGATGAAGTCACAGTCTC^{4103}-3′). The resulting PCR product was digested with *Nhe*I and *Sph*I and cloned into the prepared digested parental vector. In each case, the presence of the desired point mutation and the absence of random mutations were confirmed by sequencing. The mutagenic bases are in boldface and underlined. Analysis of the wild-type and mutant clones in in vitro transcription and translation experiments showed that mutation of the active-site cysteine resulted in loss of proteolytic activity, and an unprocessed product of approximately 200 kDa was the only protein detected (Fig. 4A). The identity of the 200-kDa band as the unprocessed polyprotein was confirmed by immunoprecipitation with both the N-terminal and C-terminal specific antisera (Fig. 4B). An unprocessed product of this size strongly suggests that translation initiation is from the first in-frame AUG at nt 5. In addition, the abolition of cleavage induced by a single point mutation suggests that the viral genome encodes only a single protease.

Mutagenesis of putative protease cleavage sites. Proteolytic processing of polyproteins by the 3C thiol protease has been

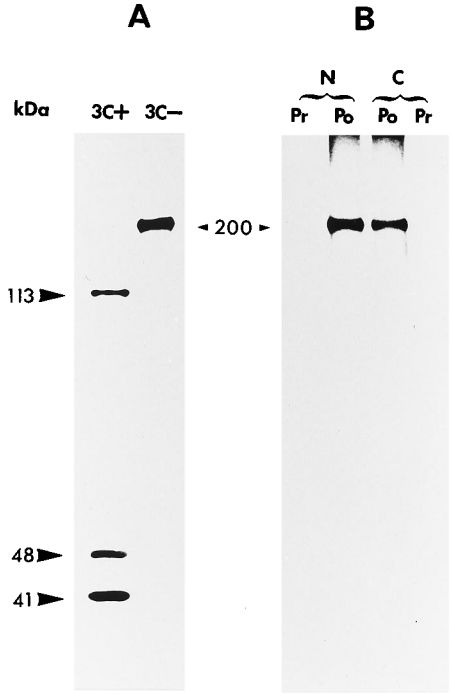

FIG. 4. In vitro transcription-translation products produced by clones of wild-type and mutant 3C-like proteases. (A) Autoradiograph of the major translation products produced by the wild type (3C+) and a Gly-1238 (3C−) mutant. The observed molecular masses of the translation products are indicated beside the lanes. (B) Immunoprecipitation of the 200-kDa mutant ORF1 protein with antisera to the N- and C-terminal polypeptides. Pr and Po refer to preimmune and immune sera, respectively.

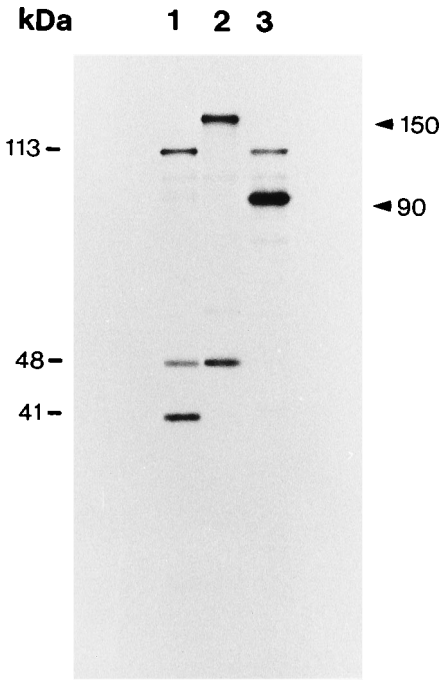

FIG. 5. SDS-PAGE of in vitro transcription-translation products produced by clones of wild-type and mutant 3C-like protease cleavage sites. The three major cleavage products produced by the wild type are shown in lane 1. Lanes 2 and 3 show the translation products produced by the two mutant clones in which a proline residue has been substituted for the glutamine residues at positions 762 and 399, respectively. The observed molecular masses are indicated beside the lanes.

studied in detail in the picornaviruses, and most of the cleavages have been shown to occur preferentially between QG and, in some cases, QS or QA amino acid pairs (29). Two conserved potential QG cleavage sites were identified in the polyprotein amino acid sequence by alignment of ORF1 of SV, NV, and Lordsdale virus. The conserved motif LQGP/K was found in SV at amino acid positions 398 (LQGP) and 761 (LQGK). Proteolytic cleavage at these positions would yield products with calculated molecular masses of 45, 40, and 114 kDa. These predicted products are very close in size to the observed proteins produced in vitro (48, 41, and 113 kDa), and since the 48-kDa protein and the larger 113-kDa protein are immunoreactive with N-terminal and C-terminal antisera, respectively, the smaller 41-kDa protein may represent the viral helicase. To test the hypothesis that the 41-kDa protein was produced by cleavage at these positions, the critical glutamine residue was converted to a proline residue by site-directed mutagenesis (Fig. 3A and B). The point mutation A-1200→C-1200 (Gln→Pro) was introduced by using the mutagenic primer SVmut3 (5′-^{1239}ATCACTGGGACTAGATCTCGTGCCAAGTC TTCAGGTCCC**G**GAAGATGG^{1192}-3′) in a PCR with primer SV107 (5′-^{953}GATAGCAAGTTAGAGTTGGT^{972}-3′). The termini of the resulting amplicon were digested with *Pst*I and *Bgl*II and cloned back into pT7SVpolyA.

The point mutation A-2289→C-2289 (Gln→Pro) was introduced with mutagenic primers by using overlap extension PCR (12, 13) as described above. The primary PCR products for the A-2289→C-2289 mutation were generated with the following primer pairs: SV30 (5′-^{1824}TGATGCTGTGGGACGACT^{1841}-3′) with SVmut5 (5′-^{2299}CATCTTTCCC**G**GTAGCTGGA A^{2279}-3′) and SVmut4 (5′-^{2279}TTCCAGCTAC**C**GGGAAAG ATG^{2299}-3′) with SV57 (5′-^{2825}GCCCATACTTTTCTACCA C^{2807}-3′). The complete fragment was generated by annealing of the two primary PCR products, followed by further rounds

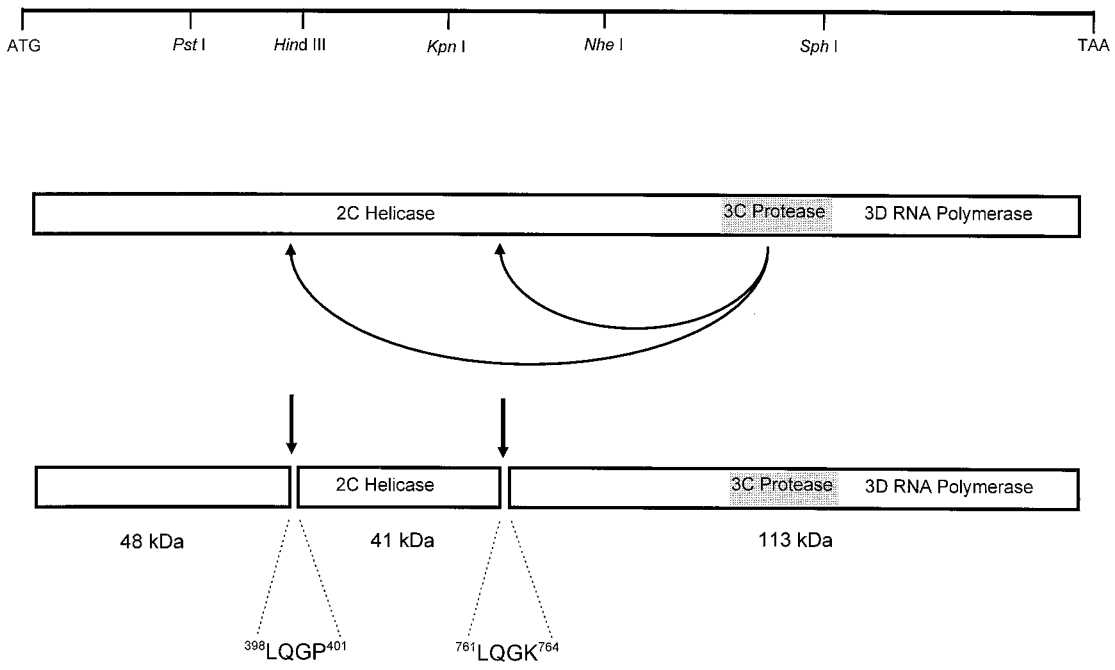

FIG. 6. Schematic diagram summarizing the observed pattern of proteolysis (arrows) of the SV ORF1 in vitro translation products. The top line represents the ORF1 region of the SV genome, and the translation products are shown as open boxes. The two 3C-like protease cleavage sites identified by site-directed mutagenesis are shown with amino acid coordinates below the putative 2C-like helicase cleavage product.

of PCR with nested primers SV108 (5′-^{2168}GGGTTTGACA ATCAAGGGA^{2186}-3′) and SV109 (5′-^{2663}AGCCAGCAATT TGTAGTATG^{2644}-3′). The resulting PCR product was digested with *Kpn*I and *Nsi*I and cloned into the prepared digested parental vector. All reconstructed clones were checked for sequence accuracy.

Elimination of the target glutamine residues by site-directed mutagenesis abolished proteolytic cleavage and production of the 41-kDa protein, further supporting the model in which this protein is the final processed form of the viral 2C-like helicase. The in vitro translation products derived from the two mutant clones is shown in Fig. 5. The results show that modification of the potential QG cleavage site abolished proteolytic processing and yielded products with molecular masses predicted precisely by the loss of one of these sites. Polyprotein processing by a proteolytic cascade is typical of the mechanism used by members of the family *Picornaviridae* (26), although these viruses do not initiate protein synthesis at the first in-frame AUG but use a cap-independent internal ribosome entry mechanism to begin translation at a site several hundred nucleotides distal to the 5′ end of the genome (1, 30).

The pattern of translation products for SV was consistent with a model of a virus-encoded protease situated upstream and adjacent to the RNA polymerase region at the C terminus of the polyprotein. Translation products from SV in an in vitro coupled transcription-translation system strongly suggest that protein synthesis is initiated at the first AUG of the polyprotein ORF and that a major cleavage product of 48 kDa is released from the N terminus. A summary of the observations is shown in Fig. 6. The fact that further processing of the 113-kDa product was not observed in vitro suggests that proteolytic cleavage of the 3C-3D region of the polyprotein occurs at a much slower rate or that host cell proteases might be involved in the production of the final forms of the nonstructural proteins. Very slow cleavage of 3CD^{pro} was observed in poliovirus, suggesting that because the polymerase moiety of 3CD^{pro} is inactive, this precursor is required for functions other than those carried out by the individual components (11). Resistance to proteolysis was also observed at the protease-polymerase boundary in rabbit hemorrhagic disease virus 3CD^{pro} (3). The SV 113-kDa 3CD^{pro} precursor is considerably larger than the picornaviral protein (approximately 72 kDa). Assuming that the 3C-like proteases are similar in size, the SV precursor protein presumably carries additional nonstructural viral proteins, such as the VPg equivalent protein found in animal caliciviruses (4, 24, 27).

The absence of a cell culture system for these fastidious viruses has severely hampered studies on the molecular biology of human caliciviruses. Thus, the use of authentic recombinant cDNA in vitro or transfection of eukaryotic cells with genomic RNA is currently the only way forward to understand the replication of these viruses.

We thank the Wellcome Trust for financial support. Binlei Liu was supported by a Sino-British Friendship Scholarship from The British Council and a University of Southampton Research Studentship.

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