Cleavage Map and Proteolytic Processing of the Murine Norovirus Nonstructural Polyprotein in Infected Cells†

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Murine norovirus (MNV) is presently the only member of the genus *Norovirus* in the *Caliciviridae* that can be propagated in cell culture. The goal of this study was to elucidate the proteolytic processing strategy of MNV during an authentic replication cycle in cells. A proteolytic cleavage map of the ORF1 polyprotein was generated, and the virus-encoded 3C-like (3CL) proteinase (Pro) mediated cleavage at five dipeptide cleavage sites, ³⁴¹E/G³⁴², ⁷⁰⁵N/⁷⁰⁶, ⁸⁷⁰E/G⁸⁷¹, ⁹⁹⁴E/A⁹⁹⁵, and ¹¹⁷⁷Q/G¹¹⁷⁸, that defined the borders of six proteins with the gene order p38.3 (Nterm)-p39.6 (NTPase)-p18.6-p14.3 (VPg)-p19.2 (Pro)-p57.5 (Pol). Bacterially expressed MNV 3CL Pro was sufficient to mediate *trans* cleavage of the ORF1 polyprotein containing the mutagenized Pro sequence into products identical to those observed during cotranslational processing of the authentic ORF1 polyprotein *in vitro* and to those observed in MNV-infected cells. Immunoprecipitation and Western blot analysis of proteins produced in virus-infected cells demonstrated efficient cleavage of the proteinase-polymerase precursor. Evidence for additional processing of the Nterm protein in MNV-infected cells by caspase 3 was obtained, and Nterm sequences ¹¹⁸DRPD¹²¹ and ¹²⁸DAMD¹³¹ were mapped as caspase 3 cleavage sites by site-directed mutagenesis. The availability of the MNV nonstructural polyprotein cleavage map in concert with a permissive cell culture system should facilitate studies of norovirus replication.

Noroviruses, members of the family *Caliciviridae*, are the major etiologic agents of nonbacterial epidemic gastroenteritis (13, 27, 30, 46, 48). The lack of a cell culture system for human pathogens has necessitated a recombinant DNA-based approach for the classification of circulating strains, the generation of diagnostic tests, and the elucidation of a proposed virus replication strategy. The human noroviruses segregate into three major genogroups (designated GI, GII, and GIV), with multiple genetic clusters, or genotypes, defined within each genogroup (2, 18, 52). The 7.6- to 7.7-kb RNA genome of the noroviruses is organized into three separate open reading frames (ORFs) (ORFs 1, 2, and 3) (17, 23). ORF1 encodes a large nonstructural polyprotein that is processed by the virus-encoded 3C-like (3CL) proteinase (Pro) into the mature nonstructural proteins (17, 23, 25). ORF2 encodes the major capsid protein, VP1, and ORF3 encodes a minor structural protein, VP2 (11, 17, 23).

In *in vitro* experiments, the human norovirus 3CL Pro recognized five cleavage sites within ORF1 with various efficiencies to release six mature cleavage products with the gene order Nterm-NTPase-p20/p22-VPg-Pro-polymerase (Pol) (4,

5, 15, 25, 26, 39). In addition, several intermediate precursor products were observed in *in vitro* studies, but their presence during norovirus replication could not be confirmed in the absence of a cell culture system (4, 25).

The recent identification of murine norovirus (MNV) and the discovery that it can be propagated in a murine macrophage-like cell line (RAW264.7 [RAW]) provided the first cell culture system to study the molecular mechanisms of norovirus replication (20, 51). The MNV (murine norovirus 1 [MNV-1]) RNA genome is 7,382 nucleotides (nt) in length, with the coding region flanked by a short, 5-nt sequence at the 5' end and a 75-nt sequence at the 3' end followed by a poly(A) tail (20). The MNV genome is organized into three ORFs with a predicted gene order identical to that of human noroviruses. However, MNV is sufficiently divergent in its sequence to warrant its segregation into a new norovirus genogroup, designated GV (20, 52). Northern blot analysis of MNV-infected cells showed the presence of two major positive-strand RNA species corresponding to the genomic and subgenomic RNA, which is characteristic of calicivirus positive-strand RNA replication (51).

The goal of our present work was to define the cleavage map of the MNV nonstructural polyprotein and examine proteolytic processing of norovirus proteins during an authentic infection in cell culture. We demonstrate that the MNV 3CL Pro can mediate the cleavage of the MNV nonstructural polyprotein *in vitro* to release six mature nonstructural proteins identical in size to their counterparts in MNV-infected cells. A decrease in the size of the N-terminal protein was observed over time in virus-infected cells, and this processing was temporally associated with the induction of apoptosis. A compar-

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ison of MNV processing with that observed in vitro for human noroviruses suggests that the study of MNV replication may lead to new insights into the conserved, as well as unique, features of replication among the members of this diverse calicivirus genus.

MATERIALS AND METHODS

Cells and virus. The murine macrophage-like cell line RAW264.7 was obtained from the ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium containing penicillin (250 units/ml) and streptomycin (250 µg/ml) and supplemented with 5% heat-inactivated fetal bovine serum. A plaque-purified isolate of MNV-1, designated MNV-1.CW1P3, was characterized previously (51) and was used as the source of virus for this study. Propagation and plaque titration assays of MNV-1 in RAW cells were carried out as described previously (51).

Lysates of MNV-1-infected cells were prepared by infecting (or mock infecting) RAW cell monolayers (5×10^7 cells) with MNV-1 at a multiplicity of infection (MOI) of 2 to 4 and incubating them at 37°C. At different times postinfection (p.i.), cells were washed with phosphate-buffered saline (PBS), scraped into fresh PBS, and pelleted by low-speed centrifugation. After centrifugation, cells were resuspended in PBS (300 µl), freeze-thawed three times, and subjected to sonication on ice (Virsonic 600; VirTis, Gardiner, NY). The protein concentration of each cell lysate was measured using the Coomassie Plus protein assay reagent (Pierce, Rockford, IL), and the lysate samples were stored at -70°C.

For radiolabeling of virus-specific proteins, RAW cell monolayers (10^8 cells) were mock infected or infected with MNV-1 at an MOI of 2 to 4 and incubated at 37°C. At 12 and 16 h p.i., cells were washed with Met- and Cys-free growth medium and incubated in the same medium for 30 min. The cells were then metabolically labeled in the presence of 2 mCi of [³⁵S]methionine (>1,000 Ci/mmol, Promix [³⁵S] in vitro cell labeling mix; Amersham Biosciences, Piscataway, NJ) in 10 ml of the methionine- and cysteine-free medium for an additional 3 h. The adherent and floating cells were collected by centrifugation, lysed in 2 ml of radioimmunoprecipitation assay buffer (37), and used in 300-µl aliquots for immunoprecipitation analysis.

Plasmid construction. Standard recombinant DNA methods were used for plasmid constructions (37). A full-length cDNA clone of the MNV-1.CW1 RNA genome was constructed as follows. One microgram of purified virus RNA was used as the template for cDNA synthesis using the SuperScript III first-strand synthesis kit and the oligo(dT) primer (Invitrogen, Carlsbad, CA) as specified by the manufacturer. The entire genome was PCR amplified using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and primers 5'-gactagttaatacagcactata GTGAAATGAGGATGGC-3', containing an *SpeI* restriction site (underlined), T7 bacteriophage RNA polymerase promoter (boldface type), and the first 16 nt (uppercase type) of the virus genome, and 5'-ataagaatgcggcgctttttttttttttttt GAAATGCATCTAACTACC-3', which contained a *NotI* restriction site (underlined), a poly(T_{21}) sequence, and the last 18 nt (uppercase type) of the genome. The PCR amplification parameters were as follows: 5 cycles of 1 min at 94°C, 1 min at 65°C, and 3 min at 72°C; 5 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C; and 22 cycles of 1 min at 94°C, 1 min at 55°C minus 0.2°C/cycle, and 3 min plus 30 s/cycle at 72°C. After digestion with *SpeI* and *NotI*, the purified fragments were ligated into *SpeI*-*NotI*-linearized pΔLac/T7-SPORT1 (4). The resulting clone, designated p20.3, contained the full-length cDNA sequence of the MNV-1 genome downstream of the T7 bacteriophage RNA polymerase promoter. Sequence analysis confirmed that the cloned genome corresponded to the consensus sequence of MNV-1.CW1 (GenBank accession number DQ285629), with the exception of a 3'-end C residue immediately upstream of the poly(A) tract that was engineered in the reverse primer sequence.

A cDNA clone of the MNV-1 ORF1, in which the first two AUG codons near the 5' end were abolished, was constructed. Forward primer 5'-GTGAATTCT AGAAGGCAACGCCATCTTCTGCGCCC-3' (corresponding to the first 35 nucleotides of the MNV-1 genome and containing mutations indicated in boldface type) and reverse primer 5'-CAAACAGTATTTACCTGGGGTGTTCGAG GC-3' (complementary to nt 5265 to 5296 of the virus genome) were used to amplify a cDNA fragment that was cloned into the pCR-XL-TOPO vector using the TOPO XL PCR cloning kit (Invitrogen). The selected clone, pΔNORF1, contained the entire MNV ORF1 and the first 241 nt of ORF2 cloned downstream from the vector T7 promoter sequence.

Selected regions of the MNV-1 genome were PCR amplified from plasmid p20.3 as a template and cloned into the bacterial expression vector pET-28a or

pET-24a (Novagen, San Diego, CA) or into the mammalian expression vector pCI (Promega, Madison, WI). (Amplified MNV-1 sequences as well as cloning vectors and their restriction sites used for cloning are listed in Table S1 of the supplemental material.) The pET-based constructs contained cloned ORF1 sequences fused to an N- or C-terminal His₆ tag to facilitate protein purification using immobilized-metal affinity chromatography (IMAC). The pCI-based expression plasmids contained genes of the individual virus proteins with engineered initiation and termination codons. Primers used in the construction and sequence analyses of the clones listed in Table S1 (see the supplemental material) are available upon request.

To analyze the processing of the C-terminal part of the ORF1 polyprotein, the ORF1 sequence beginning at nt 2565 through the 3' end of the polymerase gene was subcloned into the bacterial expression vector pET-28a in two steps. First, the intermediate construct, plasmid pMBX, was obtained as follows. The 2,031-bp BamHI-XhoI fragment from plasmid p20.3 was subcloned into the BamHI-XhoI-linearized pET-28a vector. The resulting plasmid contained an MNV-1 ORF1 sequence (nt 2565 to 4596) that was fused to the vector sequence encoding a His₆ tag under the control of the T7 promoter and that was located downstream from the bacterial ribosome-binding site. Second, to extend the polymerase sequence encoded in pMBX, the 501-bp XhoI fragment from plasmid pETMN-F (see Table S1 in the supplemental material) was ligated into the XhoI-linearized pMBX vector. Bacterial clones selected after transformation were screened by restriction enzyme digestion and by sequence analysis for the desired orientation of insertion. The resulting clone, pMBN, contained the ORF1 sequence beginning at nt 2565 through the 3' end of the polymerase gene, which was fused to the vector sequence encoding a His₆ tag at the C terminus of the recombinant protein.

To analyze processing by virus Pro at the junction of the NTPase and p18 protein sequences, the corresponding region of the ORF1 polyprotein (amino acids [aa] 683 to 744) was fused in frame at its C terminus to the Pro sequence and expressed in bacteria. In order to clone the NTPase-p18 junction sequence upstream of Pro, a DNA fragment containing the Pro sequence was amplified from plasmid p20.3 using sense primer 5'-tataaattattagcttGCCCCAGTCTCCA TCTGGTCCCGTG-3' and antisense primer 5'-taaaattatttctcagtcgcccgtacta gtgtgtgtgtgtgtgtgtCTGGAAGTCCAGAGCCTCAAG-3'. The sequence of the first oligonucleotide contained 25 nt corresponding to the beginning of the Pro gene (nt 2988 to 3012) (uppercase type) and included a HindIII site (underlined). The second oligonucleotide corresponded to the sequence complementary to the end of the Pro gene (nt 3516 to 3536) (uppercase type) and included two engineered termination codons (boldface type) and *NotI* and *XhoI* sites (underlined). To amplify the DNA fragment corresponding to the NTPase-p18 junction sequence, the following pair of primers was employed: 5'-aaatattatagg atccaGGCGCCACTGCTCTTTGCGCG-3' (sense) and 5'-tattataatattagcttGGT GCACCCATTGCTTGCACCC-3' (antisense). The sequence of the first oligonucleotide contained 21 nt corresponding to nt 2052 to 2072 of the MNV genome (uppercase type) and included a BamHI site (underlined). The second oligonucleotide contained the sequence complementary to nt 2214 to 2237 (uppercase type) and a HindIII site (underlined). The purified PCR fragments were treated with HindIII and *XhoI* and BamHI and HindIII, respectively, and ligated into BamHI-XhoI-linearized pET-28b. The resulting plasmid, designated pF2R2Pro, was verified by sequence analysis.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. (Sequences of the sense primers employed in PCR mutagenesis are given in Table S2 in the supplemental material.) The corresponding antisense primers had the precise reverse and complementary sequences. The selected clones were verified by sequence analysis.

Bacterial expression of recombinant proteins, purification, and production of region-specific antiserum. Expression of recombinant proteins in bacterial cells was performed using the *Escherichia coli* BL21(DE3) cell expression system (Novagen), and proteins were purified by the IMAC method using Ni-nitrilotriacetic (NTA) agarose (QIAGEN, Valencia, CA) as described previously (4, 42). The purified proteins expressed for the generation of the region-specific antisera were dialyzed twice against a 1,000× volume of PBS and used for immunization of guinea pigs as described previously (45). The soluble recombinant Pro (produced for use in the in vitro *trans* cleavage assay) was dialyzed against a solution containing 10% glycerol, 50 mM NaCl, 1 mM dithiothreitol, and 20 mM Tris-HCl (pH 8.0); aliquoted; and stored at -70°C.

In vitro translation assay. In vitro synthesis of ³⁵S-radiolabeled proteins was accomplished using coupled transcription and translation (TNT) systems, the TNT T7 Coupled Reticulocyte Lysate system or the TNT T7 Coupled Wheat Germ Extract system (Promega). The 50-µl reaction mixtures contained 1 to 5

μg of the desired plasmid DNAs. Radiolabeling was performed in the presence of [³⁵S]methionine (>1,000 Ci/mmol; Amersham Biosciences).

Immunoprecipitation and Western blot analysis. Immunoprecipitation assays of radiolabeled virus proteins from infected cell lysates or in vitro translation mixtures were carried out using region-specific antisera as described previously (44, 45).

Western blot analysis was performed according to standard techniques (37). Briefly, each cell lysate sample (10 μg) was solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 2.5% β-mercaptoethanol, heated at 95°C for 5 min, and subjected to SDS-PAGE separation in a 4 to 20% Tris-glycine gel (Cambrex, Rockland, ME). The resolved proteins were transferred onto a nitrocellulose membrane (Invitrogen) by electroblotting, and the membrane was incubated with region-specific antiserum. The binding of the primary antibodies was detected using goat anti-guinea pig immunoglobulins conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) followed by development with SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer's protocol. Detection of the His₆-tagged proteins expressed in bacteria was performed using Penta-His antibody (QIAGEN) and goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (Pierce) as a secondary antibody.

Nucleotide and protein sequencing. Nucleotide sequence analysis of the constructed plasmid DNAs was performed by using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit and an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA).

Direct N-terminal sequence analyses of radiolabeled virus-specific proteins and proteins expressed in bacteria were conducted as described previously (42, 44).

Caspase cleavage. Caspase cleavage assays were carried out using mouse recombinant caspase 3 (EMD Biosciences, La Jolla, CA). Briefly, 1 unit of caspase 3 was added to radiolabeled Nterm protein in buffer containing 50 mM HEPES (pH 7.4) and 10 mM dithiothreitol, and the reaction mixture was incubated at 37°C for 1 to 3 h.

RESULTS

Immunoprecipitation analysis of MNV-1 ORF1 polyprotein cleavage products with region-specific antisera. The ORF1 nonstructural polyproteins of human norovirus strains Southampton virus (SHV), Norwalk virus (NV), Camberwell virus (CV), and MD-145 virus (MDV) have been reported to undergo proteolytic processing at five cleavage sites that would yield six mature cleavage products designated Nterm, NTPase, p18 (p22), VPg, Pro, and Pol (4, 5, 15, 25, 26, 39, 40). In addition, several precursor proteins that represent intermediate forms of polyprotein processing have been described previously (4, 5, 15, 25, 39, 40). The MNV-1 ORF1 polyprotein is 1,687 aa in length and shows 59 to 64% similarity with the corresponding polyproteins of SHV, NV, CV, and MDV.

An alignment of the MNV-1 polyprotein with these noroviruses identified five potential cleavage sites, ³⁴¹E/G³⁴², ⁷⁰⁵Q/N⁷⁰⁶, ⁸⁷⁰E/G⁸⁷¹, ⁹⁹⁴E/A⁹⁹⁵, and ¹¹⁷⁷Q/G¹¹⁷⁸ (Fig. 1A and see below), which would yield six mature proteins with calculated molecular masses of 38.3, 39.6, 18.6, 14.3, 19.2, and 57.5 kDa. In order to confirm this predicted cleavage map, a full-length

cDNA clone (p20.3) of the MNV-1 genome was constructed, and the ORF1 encoded in this clone was expressed in an in vitro-coupled TNT system. Several proteins with masses ranging from approximately 18 to 110 kDa were observed (Fig. 1B, lane 1). To facilitate the identification of the protein products, we generated a panel of region-specific antisera to selected parts of the MNV-1 polyprotein within the predicted borders. The full-length sequences of the predicted mature MNV-1 VPg, Pro, and ProPol proteins were successfully expressed in bacteria, whereas expression of the predicted mature Nterm, NTPase, and p18.6 proteins failed. Consequently, discrete smaller regions from the Nterm, NTPase, and p18.6 proteins were selected and expressed in bacteria (Fig. 1A; see Table S1 in the supplemental material) as His₆-tagged fusion proteins using the pET-28a plasmid (Novagen). The recombinant proteins were purified from the insoluble fractions of the bacterial cells by the IMAC purification procedure using Ni-NTA agarose columns (QIAGEN) to at least 90% homogeneity (Fig. 1A). Guinea pigs were immunized with each protein, and the resulting antisera were shown to be antigen specific by Western blot analysis, with the exception of serum raised against NTPase (data not shown). The recombinant protein derived from the cells expressing pΔNTPase was not immunogenic (data not shown).

The ORF1 region-specific antisera were used to localize the coding sequences for individual nonstructural proteins in the MNV-1 ORF1 sequence (Fig. 1B). The anti-Nterm serum, raised against the extreme N-terminal region of ORF1, recognized three major proteins (two migrating as a 45-kDa doublet and one at 39 kDa) (Fig. 1B, lane 3). Translation of the ORF1 following elimination of the first two AUGs (construct pΔNORF1) led to the disappearance of the 45-kDa doublet band and efficient production of the 39-kDa (ΔNNterm) protein (Fig. 1B, lane 12), indicating that the three proteins that immunoprecipitated with the anti-Nterm serum were products of initiation at three different AUGs. The predicted C-terminal border of these products would define Nterm proteins with molecular masses of 33.2, 38, and 38.3 kDa. The observed discrepancy between the predicted and observed molecular masses of these proteins could be explained by either abnormal mobility in SDS-PAGE or the utilization of an unexpected cleavage site to release the C terminus of the Nterm protein. Other predicted individual proteins precipitated by region-specific sera were an 18-kDa protein with anti-p18 (Fig. 1B, lane 5), a 19-kDa protein (Pro) with anti-Pro and anti-ProPol (Fig. 1B, lanes 9 and 11), and a 57-kDa protein (Pol) with anti-ProPol (Fig. 1B, lane 11).

Larger proteins representing ORF1 processing intermedi-

cleavage products are shown. The cDNA fragments engineered for expression in *E. coli* are depicted as dark gray bars. Fusion proteins corresponding to the selected ORF1 regions were purified using Ni-NTA agarose. For lanes 1 to 6, a sample of each region-specific protein (identified above the lanes) was analyzed by SDS-PAGE followed by Coomassie blue staining. (B) Immunoprecipitation of the MNV ORF1 nonstructural proteins synthesized in a TNT reaction from the genomic full-length clone p20.3 using a panel of region-specific antisera. Radiolabeled MNV ORF1 polyprotein cleavage products (lane 1) were immunoprecipitated with either pre- or postimmunization sera raised against Nterm protein (lanes 2 and 3), p18 protein (lanes 4 and 5), VPg protein (lanes 6 and 7), Pro protein (lanes 8 and 9), and ProPol protein (lanes 10 and 11) and subjected to SDS-PAGE in a 12% Tris-glycine gel followed by autoradiography of the dried gel. Lane 12, TNT products derived from the MNV ORF1 clone pΔNORF1 in which the first two AUG codons were abolished. Proteins that were not identified in this study and that likely represented nonspecific products in the TNT reaction are indicated with asterisks.

ates were precipitated with region-specific sera. The antisera to the p18, VPg, Pro, and ProPol regions precipitated a 110-kDa protein from the *in vitro* translation mixture derived from p20.3 (Fig. 1B, lanes 5, 7, 9, and 11). Three sera, anti-VPg, anti-Pro, and anti-ProPol, precipitated a protein of approximately 90 kDa, while anti-p18 serum did not recognize this protein but precipitated one protein with a molecular mass of 18 kDa. Anti-Pro and anti-ProPol sera both recognized a 77-kDa protein, whereas anti-p18 and anti-VPg sera did not. Thus, the 110-kDa protein was likely a precursor to the 18- and 90-kDa proteins and represented the p18.6-VPg-Pro-Pol protein complex. Furthermore, the 90-kDa protein corresponded to the VPg-Pro-Pol precursor, and the 77-kDa protein corresponded to the ProPol precursor. A 14-kDa band corresponding to the mature VPg protein was not observed among translational products precipitated with the anti-VPg serum, a finding which was likely due to the absence of methionine residues in this protein sequence.

All four sera (anti-p18, anti-VPg, anti-Pro, and anti-ProPol) precipitated 55-, 45 (migrating as a doublet)-, 39-, and 35-kDa proteins (Fig. 1B, lanes 5, 7, 9, and 11). The 35- and 55-kDa proteins were detected only as faint bands in products precipitated by the anti-Nterm serum (serum specific to the N terminus of the ORF1 polyprotein), and the observed mass of these proteins was consistent with those of p18-VPg and p18-VPg-Pro, respectively. A coprecipitation of the three forms of the Nterm protein (45-kDa doublet and 39 kDa) was observed under these experimental conditions with the anti-p18, anti-VPg, anti-Pro, and anti-ProPol sera. This observation suggests possible interactions of the Nterm protein with the p18.6, VPg, Pro, and Pol proteins. These interactions would also be consistent with the detection of the p18-VPg-Pro-Pol (110-kDa) protein among the products precipitated by the anti-A serum. The possibility of such interactions among the nonstructural proteins has been reported recently for feline calicivirus (FCV) (19).

None of the region-specific sera recognized the 40-kDa protein from the p20.3 translational mixture. The identity of the 40-kDa protein as the NTPase was determined by mutagenesis and microsequencing experiments (see below).

Mapping of VPg, 3CL Pro, and polymerase. Cleavage of the calicivirus nonstructural polyprotein has been associated with a 3CL proteinase encoded in the C-terminal part of the ORF1 polyprotein (25, 34, 45, 49). Comparison of the C-terminal part of the MNV-1 ORF1 polyprotein amino acid sequence with those of CV, MDV, NV, and SHV revealed the presence of a ¹¹³¹GDCG¹¹³⁴ motif in the MNV-1 polyprotein (see Fig. S1 in the supplemental material) that included a cysteine residue that was previously shown to be part of the catalytic site (32, 41). Substitution of the Cys¹¹³³ with Ala in a full-length genomic MNV-1 clone (p20.3Pro^m) (see Table S2 in the supplemental material) abolished autocatalytic processing of the ORF1-encoded polyprotein (see Fig. S1 in the supplemental material), thus confirming the identity of the MNV-1 enzyme as a cysteine 3CL Pro.

To verify the cleavage sites bordering the MNV-1 Pro sequence, the ORF1 sequence localized between the BamHI (2,565 nt) restriction site and the ORF1 3' end was subcloned to generate clone pMBN. The protein sequence encoded in pMBN overlapped the entire VPg, Pro, and Pol and contained

a partial sequence of p18 that should yield an expressed protein of 97.3 kDa (Fig. 2A). However, comparative SDS-PAGE analysis of the proteins expressed by bacteria carrying the pMBN plasmid (Fig. 2B, lane 4) with those from the pET-28a control (Fig. 2B, lane 2) showed the presence of three unique proteins of 58, 19, and 16 kDa in the lysate of pMBN-transformed IPTG (isopropyl- β -D-thiogalactopyranoside)-induced cells. Western blot analysis of the corresponding cell lysates with region-specific antisera showed that the 16-kDa protein band was specifically recognized by anti-VPg serum and that the 19-kDa band was recognized by anti-Pro serum (Fig. 2B, lane 8). N-terminal sequence analysis of the 16-kDa protein identified the sequence GKKKGKNKK, which was consistent with the utilization of the cleavage site at position ⁸⁷⁰E/G⁸⁷¹ to release the N terminus of VPg (Fig. 2A).

To identify the 58-kDa protein, we purified the expressed protein from the soluble fraction of the bacterial cells using the IMAC procedure (data not shown) and subjected it to N-terminal sequence analysis. The identified sequence, PMLPR PSG, corresponded to aa 1180 to 1187 of the ORF1 polyprotein and was located two residues downstream from the ¹¹⁷⁷Q/G¹¹⁷⁸ cleavage site that was predicted to release the N terminus of the polymerase (Fig. 2C). Norovirus 3CL Pro has been reported to recognize cleavage sites that contain glutamine or glutamic acid residues in the P1 position (4, 5, 15, 25, 26, 39, 40). The observation of an unusual cleavage product with a Pro residue in the P1 and P1' positions suggested the presence of contaminating protease activity in the bacterial cell lysates. However, the use of a protease inhibitor cocktail during the protein purification procedure did not prevent this aberrant processing (data not shown). To establish whether the same N terminus is generated in a eukaryotic expression system, we expressed the pMBN sequence in a TNT T7 system. Microsequencing performed on the *in vitro*-synthesized 58-kDa protein radiolabeled with [³⁵S]methionine demonstrated a peak of radioactivity for residue 4 (Fig. 2C), which was consistent with cleavage of the ORF1 polyprotein at the ¹¹⁷⁷Q/G¹¹⁷⁸ site (Fig. 2C). Furthermore, mutagenesis of the Gln¹¹⁷⁷ residue to Gly in the p20.3 clone abolished expression of the 58-kDa protein (data not shown).

We cloned and expressed the sequence of the ORF1 polyprotein bordered by the newly determined ⁸⁷⁰E/G⁸⁷¹ and ¹¹⁷⁷Q/G¹¹⁷⁸ cleavage sites (pMVP) (see Table S1 in the supplemental material) in bacteria (Fig. 2A). Two bands corresponding to proteins with molecular masses of approximately 16 and 19 to 20 kDa (Fig. 2B, lane 6) comigrated with the analogous proteins from pMBN-expressing cells and were recognized by anti-VPg and anti-Pro sera (Fig. 2B, lane 9). A His₆ tag sequence introduced at the C terminus of the pMVP-cloned sequence facilitated the purification of the 19- to 20-kDa protein from the soluble fraction of the bacterial cells. Direct sequence analysis of the purified protein identified the sequence APVSI at its N terminus, consistent with the presence of a cleavage site at position ⁹⁹⁴E/A⁹⁹⁵ that would define the border between the VPg and Pro proteins in the ORF1 polyprotein. Cleavage site ⁹⁹⁴E/A⁹⁹⁵ together with ¹¹⁷⁷Q/G¹¹⁷⁸ defined the boundaries of MNV-1 Pro with a calculated molecular mass of 19.2 kDa, while sites ⁸⁷⁰E/G⁸⁷¹ and ⁹⁹⁴E/A⁹⁹⁵ defined the boundaries of MNV-1 VPg with a calculated molecular mass of 14.3 kDa (Fig. 2A).

FIG. 2. Mapping of VPg, 3CL Pro, and polymerase. (A) Schematic representation of the clones used to express VPg-, Pro-, and Pol-containing ORF1 polyprotein sequences in bacteria. Observed molecular masses of the detected cleavage products used in the direct N-terminal protein sequence analysis are shown at the top of the diagram, and identified cleavage sites are shown at the bottom of the diagram. (B) SDS-PAGE and Western blot analysis of the MNV-specific proteins encoded in plasmids pMBN and pMVP and expressed in bacteria. Bacterial cells carrying either the pMBN or pMVP plasmid or the pET-28a vector plasmid were induced with IPTG, and lysates of the cells were prepared as described in Materials and Methods. Aliquots of the bacterial cell lysates were subjected to SDS-PAGE and visualized with Coomassie blue stain. Lanes 1, 3, and 5, uninduced cells harboring pET-28a, pMBN, and pMVP, respectively; lanes 2, 4, and 6, induced cells harboring the corresponding plasmids. Expressed recombinant proteins are indicated with arrows. To confirm the identity of the 16-kDa protein as VPg and the 19-kDa protein as proteinase, Western blot analysis using anti-VPg and anti-Pro sera was performed on lysates of induced bacterial cells harboring pET-28a (lane 7), pMBN (lane 8), and pMVP (lane 9). N-terminal sequence analysis of the VPg protein was performed using a protein that was SDS-PAGE purified from the lysate of IPTG-induced cells harboring pMBN. His₆-tagged full-length polymerase (Pol-His) and proteinase (Pro-His) proteins were purified from soluble fractions of cells carrying pMBN and pMVP, respectively, for direct N-terminal sequence analysis using IMAC chromatography. MW, molecular weight (in thousands). (C) Profile of radioactivity released during Edman degradation of the [³⁵S]methionine-labeled Pol-His protein derived in vitro from pMBN. Coupled transcription and translation of pMBN were performed in the TNT system in the presence of [³⁵S]methionine. The 58-kDa protein (Pol-His) was electrotransferred onto a polyvinylidene difluoride membrane following SDS-PAGE and subjected to microsequencing. The released radioactivity was determined for each cycle and is plotted against the residue number. The predicted N-terminal sequence of Pol-His is shown below the histogram, and the mapped cleavage site is indicated with arrow. The first amino acid residue of Pol-His produced in bacteria is denoted with a circle.

Cloning and expression of the MNV-1 3CL Pro sequence (pPro) (Fig. 1A; see Table S1 in the supplemental material) in bacteria resulted in the synthesis of an active proteinase that cleaved the p20.3Pro^m in vitro-derived polyprotein into polypeptides with observed molecular sizes identical to those produced by autocatalytic cotranslational processing of the ORF1 polyprotein derived from p20.3 (see Fig. S1 in the supplemental material).

Mapping the “3A-like” protein p18.6. To define the N terminus of the 18-kDa protein, which was shown by immunoprecipitation analysis to be localized upstream of the VPg gene, we aligned the MNV-1 ORF1 polyprotein sequence corresponding to the putative border between the 18-kDa and VPg proteins with the corresponding regions from the CV, MDV, NV, and SHV polyproteins (Fig. 3A). The alignment showed the presence of dipeptide ⁷⁰⁵Q/N⁷⁰⁶ as a possible cleavage site in the MNV-1 ORF1 polyprotein sequence that might be recognized by virus 3CL Pro. In order to verify this cleavage site, we constructed plasmid pF2R2Pro, in which a 62-aa sequence

overlapping the putative junction between the NTPase and 18-kDa proteins was fused to the His₆-tagged sequence of MNV-1 Pro (Fig. 3B). The chimeric protein with a calculated mass of 30 kDa was expressed in both in vitro (TNT) and bacterial expression systems. Analysis of products derived from this plasmid in both expression systems indicated the presence of proteolytic activity resulting in the partial cleavage of the encoded protein (Fig. 3C, lanes 1 and 2). The 24-kDa cleavage product was shown by Western blot analysis to contain His₆-tagged and Pro sequences (Fig. 3C, lanes 3 and 4). The 24-kDa protein purified by IMAC and SDS-PAGE was subjected to direct N-terminal sequencing analysis. The analysis identified the sequence NKVYDFDAG, consistent with cleavage at ⁷⁰⁵Q/N⁷⁰⁶ of the ORF1 polyprotein. Sites ⁷⁰⁵Q/N⁷⁰⁶ and ⁸⁷⁰E/G⁸⁷¹ defined the borders of the p18 (“3A-like”) protein with a calculated molecular mass of 18.6 kDa.

Mapping the NTPase and Nterm proteins. Alignment of norovirus ORF1 polyprotein sequences showed the presence of several conserved motifs in the region corresponding to the

FIG. 4. Analysis of the cleavage site between Nterm and NTPase. (A) Alignment of the amino acid sequence of the putative junction of the MNV Nterm and NTPase proteins with those of CV, MDV, NV, and SHV. Numbers correspond to the ORF1 polyprotein sequence of each virus, and asterisks indicate conserved amino acids. (B) Comparative analysis of the MNV ORF1 polyprotein processing products synthesized in vitro from full-length genomic clone p20.3 (lane 1) and its derivative, p20.3m341, carrying an Nterm-NTPase cleavage site mutation (³⁴¹EG→³⁴¹AG) (lane 3). Lanes 2 and 4, in vitro-radiolabeled proteins derived from clones pCINTerm and pCINTPase, encoding predicted sequences of Nterm (aa 1 to 341) and NTPase (aa 342 to 705), respectively.

with the beginning of the NTPase sequence at Gly³⁴² (data not shown). Finally, indirect confirmation of the borders of the NTPase protein defined by ³⁴¹E/G³⁴² and ⁷⁰⁵Q/N⁷⁰⁶ was obtained when the corresponding sequence was subcloned into the pCI vector under the control of the T7 RNA polymerase promoter and when the mobility of the protein derived from the resulting plasmid, pCINTPase, was found to be identical to that of the 40-kDa protein generated by proteolytic processing of the ORF1 polyprotein (Fig. 4B, lane 2). In addition, in vitro expression of the first 341 aa of the MNV-1 ORF1 subcloned into pCINterm (corresponding to the Nterm protein) resulted in the synthesis of a protein with an observed mobility of 45 kDa (calculated molecular mass of 38.3 kDa) that comigrated with its Nterm counterpart from p20.3 translational products (Fig. 4B, lane 4).

Analysis of MNV-1 nonstructural protein expression in virus-infected RAW cells. To investigate the profile of MNV-1

nonstructural protein expression during virus replication, we prepared total lysates of MNV-infected (MOI of 2 to 4) cells at 4-h time intervals p.i. Western blot analysis of these samples with mouse MNV-1 infection serum showed the accumulation of proteins at various times, with detection at 12 h p.i. and peak synthesis at 12 to 16 h (Fig. 5A). The predominant proteins detected with this serum were 43, 45, 60, and 115 kDa in size. A reduction in the intensity of the 60- and 45-kDa protein bands was observed as the time course progressed, while the amount of 43-kDa protein increased starting at 12 h p.i. The maximum amount of 43-kDa protein was detected at 20 h p.i. (Fig. 5A). Of note, the efficiency of detection of the 115-kDa protein varied among experiments. In order to further elucidate the identities of proteins in the infected cell lysates, we performed Western blotting with the region-specific sera and with serum raised against the CsCl-purified MNV-1 virions (51). Furthermore, the proteins were compared with mature

A.

B.

C.

D.

E.

F.

G.

H.

FIG. 5. Expression of MNV-1 proteins in infected cells. (A to C and E to H) Virus proteins in MNV-1-infected cells collected at different time points following infection were analyzed. Ten micrograms of each cell lysate prepared from collected cells was resolved on a 4 to 20% polyacrylamide gel by SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. The membrane was probed with serum collected from a mouse that had undergone infection with MNV-1 (A). The same lysates from the MNV-infected cells were probed with region-specific antisera: anti-capsid (B), anti-Nterm (C), anti-ProPol (E), anti-Pro (F), anti-p18 (G), and anti-VPg (H) sera. To compare the mobilities of the virus proteins synthesized in infected cells and in vitro, TNT products derived from pCIORF2, pCIPol, pCIPro, and pCINterm were analyzed concurrently with the corresponding cell lysate samples probed with anti-capsid (B, lane 8), anti-Nterm (C, lane 8), and anti-ProPol (E, lane 8) sera. (D) Comparison of MNV Nterm proteins expressed in vitro and in infected cells. Immunoprecipitation (IP) of the radiolabeled Nterm protein synthesized in the in vitro system from pCINterm and p20.3 (lanes 1 and 3, respectively) and in infected cells (lane 2) is shown. Lane 4, the MNV ORF1 nonstructural proteins derived from p20.3 in vitro. (I) Comparative immunoprecipitation analysis of p18-, VPg-, and Pro-related proteins derived by in vitro cleavage of the ORF1 polyprotein or synthesized in MNV-infected cells. The radiolabeled p18-related proteins were precipitated using anti-p18 serum from the ORF1 (lane 1) and pCIP18 (lane 3) translation mixture or from MNV-infected cells (lane 2). The radiolabeled VPg-related proteins were precipitated using anti-VPg serum from the ORF1 translation mixture (lane 4) or from MNV-infected cells (lane 5). The radiolabeled Pro-related proteins were precipitated using anti-Pro serum from the ORF1 (lane 6) and pCIPro (lane 8) translation mixture or from MNV-infected cells (lane 7). (J) Expression of the p18 protein in in vitro systems. Lanes 1 and 2, immunoprecipitation of the p18 protein derived from pCIP18 using the TNT T7 Coupled Reticulocyte Lysate system (RR) or the TNT T7 Coupled Wheat Germ Extract system (WGE) (Promega), respectively; lane 3, the MNV ORF1 nonstructural proteins derived from p20.3 using the TNT T7 Coupled Reticulocyte Lysate system. Modified and free forms of the p18 protein are indicated with arrows. MW, molecular weight (in thousands).

nonstructural proteins that had been cloned into a pCI vector and expressed in the TNT system. The approximately 60-kDa band was identified as the virus capsid protein VP1, because it was recognized by serum raised against purified MNV-1 virions (51) (Fig. 5B, lanes 3 to 7), and its mobility was similar to that of VP1 derived by in vitro translation from a plasmid (pCIORF2) carrying the entire ORF2 of the MNV-1 genome cloned under the control of the T7 RNA polymerase promoter (Fig. 5B, lane 8). The similarity in the observed mobilities of these proteins indicates that the MNV-1 capsid protein does not undergo proteolytic processing during its maturation. Of interest, the anti-MNV-1 virion serum recognized also a 120-kDa protein that was consistent in size with a dimeric form of the MNV-1 capsid protein (Fig. 5B, lanes 4 and 5). Western blot detection of capsid protein dimers has previously been described for FCV and rabbit hemorrhagic disease virus (RHDV) (24, 44).

As shown in Fig. 5C, using anti-Nterm serum, we identified

proteins with observed masses of 43, 45, and 115 kDa in MNV-1-infected cells as Nterm-related proteins. The mobility of the 45-kDa protein in cells corresponded to that of the Nterm protein expressed from pCINterm in the TNT system, indicating that the 45-kDa protein was the full-length Nterm protein (Fig. 5C, lanes 4 to 8, and D, lanes 1 to 4). The detected change in the relative amounts of the 45- and 43-kDa proteins in the Nterm Western blot was similar to that observed with the infection serum and suggested a modification of the Nterm protein over time. The presence of the 115-kDa protein could reflect either the existence of a larger precursor that would include sequences of p18 and possibly VPg or the existence of multimeric forms of Nterm or an Nterm-NTPase complex. However, the probing of cell lysates with anti-p18 serum resulted in the detection of only the p18 protein (Fig. 5G, lanes 4 to 7). The characterization of the 115-kDa complex will require further study.

The predominant form of the virus polymerase recognized

in infected cells by anti-ProPol serum was a 57-kDa protein (Fig. 5A and E, lanes 3 to 7). The detected protein had the same mobility as a polymerase protein derived in vitro from plasmid pCIPol (Fig. 5E, lane 8) that contained the C-terminal ORF1 sequence bordered by the cleavage site ¹¹⁷⁷Q/G¹¹⁷⁸. Of interest, the anti-ProPol serum failed to detect the 19-kDa Pro in the Western blot, which differed from the immunoprecipitation data obtained in in vitro expression experiments and shown above (Fig. 1B, lane 11). In contrast, antiserum raised against the entire recombinant Pro reacted with the 19-kDa protein in both the Western blot assay (Fig. 5F, lanes 4 to 7) and the immunoprecipitation assay (Fig. 1B, lane 9). Neither anti-ProPol nor anti-Pro serum was found to detect significant amounts of the 77-kDa ProPol precursor in the cell lysates, suggesting that efficient cleavage of the MNV-1 ORF1 polyprotein at the border between virus Pro and polymerase proteins occurs during virus infection.

An unusual pattern of expression was observed in the immunoblot analysis of the VPg protein. Probing of membranes with anti-VPg serum revealed the presence of several faint bands corresponding to proteins with molecular masses ranging from 35 to 110 kDa (Fig. 5H) and two major bands corresponding to proteins with molecular masses of approximately 16 and 18 kDa that peaked in intensity at 12 and 16 h p.i. (Fig. 5H, lanes 4 and 5). However, the VPg protein bands were not detected at later time points (20 and 24 h p.i.). Multiple forms of VPg were found in FCV-infected cells and were proposed to represent RNA-linked VPg molecules and VPg precursor proteins (43). The finding of two MNV-1 VPg forms with low molecular masses might also reflect a modification of this protein in MNV-1-infected cells. Moreover, the disappearance of these bands with the progression of virus replication suggests a coordination between the role of VPg as a nonstructural protein in RNA replication and its packaging into mature virions via its covalent linkage of genomic RNA. It is of interest that the decrease in VPg correlated with the decrease in capsid protein retained inside cells at 20 to 24 h p.i. (Fig. 5B). This is the time interval at which peak amounts of infectious virus are detected in the cell culture medium (51).

To further investigate whether the VPg protein was present during virus replication in the form of larger precursor proteins, we conducted immunoprecipitation analysis of [³⁵S]methionine-labeled proteins from the MNV-1-infected cells using anti-p18, anti-VPg, and anti-Pro sera. Immunoprecipitation was carried out under more stringent (0.3% SDS) binding/washing conditions than those used for the analysis of in vitro-synthesized proteins described above (Fig. 1B). Again, the anti-VPg serum recognized larger precursor proteins identified above as p18-VPg-Pro-Pol, VPg-Pro-Pol, and p18-VPg-Pro (Fig. 5I, lane 5). p18-VPg-Pro-Pol and p18-VPg-Pro proteins were also precipitated by anti-p18 serum (Fig. 5I, lane 2). Both sera recognized the 35-kDa protein, suggesting the presence of a p18-VPg precursor (Fig. 5I, lanes 2 and 5). Of interest, proteins corresponding in size to the Nterm proteins of 45 and 39 kDa were again precipitated by both sera from products generated in vitro from p20.3 (Fig. 5I, lanes 1 and 4), but the amounts of these proteins precipitated from infected cells were significantly reduced under the increased stringency of the washing conditions (Fig. 5I, lanes 2 and 5).

The predominant form of 3CL Pro precipitated from the

infected cells was a 19-kDa protein. This protein had the same mobility as Pro produced in vitro by the processing of the ORF1 polyprotein or as a protein expressed individually from the corresponding ORF1 sequence (aa 995 to 1177) subcloned into the pCI vector (Fig. 5I, lanes 6 to 8). Anti-Pro serum also recognized the putative p18-VPg-Pro precursor in infected cells (Fig. 5I, lane 7). The larger precursors, p18-VPg-Pro-Pol, VPg-Pro-Pol, and Pro-Pol, were detected as faint bands with a longer gel exposure time (data not shown).

It should be noted that expression of the individually subcloned p18 sequence (pCIP18) resulted in the synthesis of two proteins, one of the expected size (18 kDa) that corresponded to the protein immunoprecipitated from infected cells or ORF1 TNT products and one with a slower mobility, designated p18* (Fig. 5J, lane 1). In order to address the possibility that an unknown modification of the p18 protein had occurred during its synthesis in rabbit reticulocyte lysates, the same construct was analyzed using the TNT T7 Coupled Wheat Germ Extract system (Promega). Of interest, this translation system yielded only the expected p18 protein (Fig. 5J, lane 2).

Caspase 3 cleaves the virus Nterm protein. Because the N-terminal regions of other caliciviruses such as FCV, RHDV, and human sapovirus Mc10 undergo proteolytic processing to generate two smaller proteins (31, 34, 42), the different forms of the MNV-1 Nterm protein in infected cells were investigated further. The Western blot analysis described above (Fig. 5C, lane 6) showed that the 43-kDa form of the Nterm protein was predominant at 20 h p.i. An analysis by immunoprecipitation of proteins produced in MNV-infected cells at 20 h p.i. again showed the predominance of the 43-kDa protein (Fig. 6A, lane 2). In addition, minor bands corresponding to proteins of 30, 21, and 17 kDa were detected (Fig. 6A, lanes 1 to 3).

The identities of these smaller proteins were examined in a series of experiments in which the intact Nterm protein produced in a TNT reaction was incubated with various preparations that might contain the protease responsible for the cleavage. First, treatment of the in vitro-synthesized Nterm protein with purified recombinant MNV-1 Pro in a *trans* cleavage assay did not generate the 43-kDa protein, but it did yield a unique 38-kDa protein (Fig. 6A, lane 4). However, the 38-kDa protein was not detected among virus proteins expressed in infected cells or generated in vitro by 3CL Pro-mediated processing of the ORF1 polyprotein (data not shown). Following incubation of the in vitro-synthesized Nterm protein with lysates of infected cells collected at different time points after infection, as indicated, the presence of proteolytic activity in cells collected starting from 12 h p.i. was associated with the appearance of the 17- and 30-kDa Nterm cleavage products (Fig. 6A, lanes 5 to 10). A faint band corresponding to the 43-kDa protein immunoprecipitated from infected cells was also detected among the cleavage products of the cell lysate-treated Nterm protein (Fig. 6A, lanes 6 to 10). However, an analogous protein with similar mobility was detected after treatment of the Nterm protein with a lysate of mock-infected cells (Fig. 6A, lane 6). These data suggest that the 43-kDa protein may be produced by proteolytic processing of the 45-kDa protein by a cellular proteinase.

Morphological and biochemical analysis of the RAW cells infected with MNV-1 showed that starting from 12 h p.i., the

FIG. 6. Detection and mapping of the MNV Nterm protein cleavage by caspase 3. (A) Comparison of cleavage products of the MNV Nterm protein in virus-infected cells with those generated by cleavage with the virus Pro protein and caspase 3 in vitro. Lanes 1 and 2, immunoprecipitation of the radiolabeled Nterm protein from the virus-infected cells using pre- and postimmunization anti-Nterm serum, respectively; lane 4, products of incubation of the radiolabeled Nterm protein derived by TNT translation from pCINterm (Nterm TNT) (2 μ l of the translational mixture) with recombinant virus proteinase (HisPro) (2 μ g, 3 h, 37°C), respectively (the 38-kDa cleavage product is indicated with an arrow); lanes 6 to 10, products of incubation of the Nterm TNT with lysates (10 μ l) of the cell collected at different times of infection; lane 11, incubation of the Nterm TNT with one unit of mouse recombinant caspase 3 (Cas3). Untreated Nterm TNT (incubated 3 h at 37°C) was included as a control (lanes 3 and 5). Asterisks denote cleavage products of the Nterm protein precipitated from infected cells that had mobilities similar to those generated by caspase 3 cleavage. MW, molecular weight (in thousands). (B) Schematic representation of the Nterm clones pCINtermG¹⁰³, pCINtermG¹²¹, and pCINtermG¹³¹ derived from pCINterm by site-directed mutagenesis of the caspase 3 putative cleavage sites ¹⁰⁰DMSD¹⁰³, ¹¹⁸DRPD¹²¹, and ¹²⁸DAMD¹³¹, respectively. Predicted caspase 3 cleavage products of the mutant Nterm proteins are depicted as gray boxes, and their molecular masses are shown above the diagram. (C) SDS-PAGE analysis of cleavage products obtained after incubation (3 h at 37°C) of caspase 3 (Cas3) with TNT-radiolabeled Nterm (lane 4) or with its mutant versions, NtermG¹⁰³ (lane 5), NtermG¹²¹ (lane 6), and NtermG¹³¹ (lane 7). TNT mixture without plasmid (lane 1), TNT-derived Nterm protein (lane 2), and the same protein after 3 h of incubation in assay buffer (lane 3) were included as controls.

infected cells exhibited a number of changes characteristic of apoptosis, including activation of caspases 3, 8, and 9 (S. V. Sosnovtsev, unpublished data). To test whether active caspases are responsible for the cleavage of the MNV-1 Nterm protein, we incubated in vitro-synthesized Nterm protein with recombinant human caspases 3, 8, and 9 and mouse caspase 3. Incubation with caspases 8 and 9 did not produce any cleavage

products (data not shown), while incubation with both human (data not shown) and mouse caspase 3 resulted in the detection of 30-, 28-, 18-, and 17-kDa proteins (Fig. 6A, lane 11). Of interest, the 28-kDa protein was also present among Nterm cleavage products generated by the incubation of this protein with the infected cell lysate. It comigrated with one of the proteins observed in the control sample, and it was detected

only after prolonged SDS-PAGE separation of these proteins (data not shown). It should be noted that caspase 3 treatment of the in vitro-synthesized Nterm protein did not generate the 43-kDa protein observed in infected cells. The mechanism responsible for the production of this protein will require further studies.

Examination of the Nterm amino acid sequence for a putative caspase 3 cleavage site (DXXD) showed the presence of three sequences that could be processed by this proteinase: $^{100}\text{DMSD}^{103}$, $^{118}\text{DRPD}^{121}$, and $^{128}\text{DAMD}^{131}$ (Fig. 6B). To investigate whether these sites are recognized by caspase 3, we replaced Asp residues in the P1 positions of these cleavage sites with Gly using site-directed mutagenesis and plasmid pCINterm as the template. The mutagenized Nterm proteins were expressed in the TNT system and incubated with active recombinant mouse caspase 3. SDS-PAGE analysis of the cleavage products of the protein derived from pCINtermG 103 showed a profile similar to that observed for the authentic Nterm protein, suggesting that the $^{100}\text{DMSD}^{103}$ site is not recognized by caspase 3 (Fig. 6C, lane 5). In contrast, mutagenesis of $^{118}\text{DRPD}^{121}$ and $^{128}\text{DAMD}^{131}$ sites resulted in changes in the cleavage profile (Fig. 6C, lanes 6 and 7). The D $^{121}\rightarrow$ G 121 mutation led to the disappearance of the 30-kDa protein band and the generation of a new 20-kDa protein band (Fig. 6C, lane 6). The D $^{131}\rightarrow$ G 131 substitution resulted in the disappearance of the 28-kDa protein band (Fig. 6C, lane 7). The absence of the 20-kDa and the presence of 30-kDa protein bands in cleavage products of the Nterm protein suggested more efficient recognition by caspase 3 at the $^{118}\text{DRPD}^{121}$ site. Of interest, the molecular masses of the caspase 3 cleavage products with observed mobilities of 18, 28, and 30 kDa were predicted to be 13.6, 23.7, and 24.7 kDa, respectively. The discrepancies in the predicted and observed protein SDS-PAGE mobilities were consistent with the unusual mobility of the full-length Nterm protein observed.

DISCUSSION

The replication strategy of viruses in the family *Caliciviridae* includes proteolytic processing of a large polyprotein by the virus-encoded 3CL Pro protein to release several nonstructural proteins. The 3CL Pro of MNV-1 maps to amino acids 995 to 1177 of the polyprotein and processes the polyprotein at five cleavage sites, $^{341}\text{E/G}^{342}$, $^{705}\text{Q/N}^{706}$, $^{870}\text{E/G}^{871}$, $^{994}\text{E/A}^{995}$, and $^{1177}\text{Q/G}^{1178}$, to release six proteins with the following gene order: p38.3 (Nterm)-p39.6 (NTPase)-p18.6-p14.3 (VPg)-p19.2 (Pro)-p57.5 (Pol). Despite the divergence of the ORF1 polyprotein amino acid sequences, the location of the cleavage sites as well as a sequence preference for glutamic acid or glutamine residues in the P1 position were found to be well conserved among noroviruses. In contrast, some sequence variation in the P1' position of the cleavage sites is tolerated. A comparison of the MNV-1 ORF1 sequence with those of other MNV strains in GenBank (accession numbers DQ223041, DQ223042, and DQ223043) showed variation in the P1' position at the sites $^{705}\text{Q/N}^{706}$ and $^{870}\text{E/G}^{871}$ (N $^{706}\rightarrow$ S 706 and G $^{871}\rightarrow$ S 871).

Our studies confirmed that the processing events identified in in vitro studies of MNV correlated with those observed in infected cells, with the exception of the Nterm protein. The

observed mass (45 kDa) of the MNV-1 Nterm protein detected in cells early in infection was identical to that of the full-length Nterm protein derived by cleavage of ORF1 in vitro or by in vitro expression of the first 341 aa of ORF1 (pCIM1-341). Moreover, the size of the Nterm protein in infected cells was consistent with the utilization of the first AUG of ORF1 for initiation of translation. As the infection proceeded, smaller forms (43, 30, 21, and 17 kDa) of the Nterm protein accumulated, suggesting that the protein was undergoing additional proteolytic processing. Previously, proteolytic processing of the norovirus Nterm protein had been observed only in studies of the Camberwell GII human norovirus strain in which the ORF1 polyprotein was expressed in transfected cells (40). It was previously suggested that virus proteinase might cleave this protein at a predicted $^{194}\text{E/S}^{195}$ cleavage site in a mechanism that required cellular factors, because in vitro processing of Nterm in TNT reactions had not been detected in this study or other studies (15, 25, 40). In contrast, 3CL Pro-mediated cleavage was readily observed in vitro for the Nterm region proteins of other caliciviruses, including the 16- and 23-kDa proteins of RHDV (*Lagovirus*), the 5.6- and 32-kDa proteins of FCV (*Vesivirus*), and the 11- and 28-kDa proteins of human sapovirus Mc10 (*Sapovirus*) (34, 42, 50). In addition, processed forms of the Nterm-equivalent proteins were detected in FCV (p32)- and RHDV (p16 and p23)-infected cells (21, 42). The importance of this cleavage event for the FCV replication cycle was demonstrated when the ablation of the cleavage site between the p5.6 and 32-kDa proteins in an infectious cDNA clone of FCV was shown to prevent virus recovery (42). In this study, an alignment of norovirus Nterm sequences with those of other caliciviruses failed to reveal the presence of a conserved 3CL cleavage site (data not shown). In addition, an in vitro *trans* cleavage assay did not implicate MNV 3CL Pro as the proteinase responsible for the production of the 43-, 30-, 21-, and 17-kDa proteins detected in the MNV-infected cells. The latter three proteins were likely produced by the cleavage of the MNV Nterm protein by the cellular proteinase caspase 3, which was activated at a late stage in infection when cells were undergoing apoptotic changes (Sosnovtsev, unpublished). These products could also be generated by incubation of MNV-infected cell lysates or recombinant caspase 3 with Nterm protein synthesized in vitro. It is not clear whether this caspase-specific cleavage late in infection is essential for MNV-1 replication, since RNA replication, protein synthesis, and the release of infectious virus can be detected earlier (51). However, two mapped caspase 3 cleavage sites, $^{118}\text{DRPD}^{121}$ and $^{128}\text{DAMD}^{131}$, were found to be conserved as DXXD caspase 3 recognition sites in the ORF1 sequences of the MNV strains available from GenBank (accession numbers DQ223041, DQ223042, and DQ223043). The caspase-specific cleavage of the MNV-1 Nterm protein might reflect proteolytic degradation associated with MNV-induced apoptosis of RAW cells, as described previously for the FCV capsid protein during infection (1). Similarly, the 45- to 43-kDa conversion of the Nterm protein that was detected using both Western blotting and immunoprecipitation analysis might represent additional proteolytic cleavage by an unknown cellular proteinase released (or activated) due to ongoing virus-induced intracellular changes. In addition, it remains possible that active virus rep-

lication or a cellular factor might modulate substrate recognition and activity of MNV 3CL Pro.

The Nterm protein, together with the next two proteins (NTPase and p18) in the gene order of the MNV ORF1, may be involved the formation of the virus replication complex (14, 19, 50). Despite the presence of significant divergence (up to 58% for Nterm, 34% for NTPase, and 65% for p18 among norovirus proteins), all three MNV proteins contain conserved regions of hydrophobic amino acids consistent with those of a membrane-spanning region (35, 36). These predicted regions are located in the C-terminal parts of the Nterm (aa 284 to 315) and p18 (aa 106 to 125) proteins and in the N terminus of the NTPase protein (aa 1 to 28). The identification of the putative membrane interaction domains suggests a membrane-anchoring function for these proteins during the formation of norovirus replication complexes. In agreement with this hypothesis, the FCV equivalents of the norovirus Nterm, NTPase, and p18 proteins were found to be associated with the virus membranous replication complexes (14). Targeting of the intracellular membranes may be mediated by the presence of additional signals in the sequences of these proteins. The NV Nterm 301- to 398-aa sequence was sufficient to target the transport of the chimeric enhanced green fluorescent protein to Golgi complex-associated structures (9). Moreover, Ettayebi and Hardy previously reported that the NV p48 (Nterm) protein interacts with the SNARE regulator VAP-A and suggested that this protein could play a regulatory role in intracellular vesicle transport, likely affecting post-Golgi network trafficking (8). The assembly of replication complexes might be assisted by protein-protein interactions, because Kaiser et al. previously showed that p32, the FCV analog of the norovirus Nterm protein, efficiently interacts with most of the virus nonstructural proteins, including p39 (NTPase), p30 (p18-like), and p76 (ProPol) (19).

The function of the calicivirus 3A-like protein (p18 for MNV-1) is unknown. Most of the p18 protein precipitated from MNV-infected cells was in the form of the mature protein itself. However, larger proteins, likely intermediate precursor forms, were also detected. The p18-associated precursors included p18-VPg, p18-VPg-Pro, and p18-VPg-Pro-Pol. A possible function for one or more of these precursors might involve an interaction with cellular membranes and other virus nonstructural proteins to position the VPg for its proposed role in replication as a primer for RNA synthesis (28).

The 14.3-kDa MNV-1 VPg protein is a 123-aa protein mapping between the p18 and 3CL proteinase. The VPg sequence is relatively conserved among caliciviruses; the MNV-1 VPg sequence shows 60 to 65% similarity to that of other noroviruses and 31 to 42% similarity to vesiviruses, sapovirus, and lagoviruses. The calicivirus VPg protein is covalently linked to the 5' end of the viral RNA and is associated with the initiation of translation (6, 7, 12, 16, 38). Direct interaction of VPg with eukaryotic cell translation initiation factors such as eukaryotic initiation factor 3 and eukaryotic initiation factor 4E has been demonstrated for NV and FCV proteins (6, 12).

The two major cleaved forms of VPg observed in MNV-infected cells are likely free (16-kDa) and nucleotidylated (18-kDa) forms of the protein. Two FCV VPg proteins, 13 and 15 kDa, were detected in FCV-infected cells, and the 15-kDa form was radiolabeled when cells were incubated with

[³²P]orthophosphate (43; Sosnovtsev, unpublished). Similar to the FCV VPg protein, the MNV-1 VPg protein was also detected in infected cells as part of larger precursor proteins (p18-VPg, p18-VPg-Pro, and p18-VPg-Pro-Pol) (see above). In addition to the precursors containing the p18 sequence, the VPg sequence was associated with a possible VPg-Pro-Pol intermediate. Of interest, this precursor has not been observed in *in vitro* studies of ORF1 polyprotein processing of other noroviruses (4, 5, 15, 26); however, it was expressed in mammalian cells transfected with ORF1 cDNA that contained a mutagenized cleavage site between VPg and Pro (39). The synthesis of this precursor during infection was also reported for FCV (42). Besides the proposed function of presenting VPg during virus RNA replication, the VPg precursors might play a regulatory role in RNA translation. Due to the binding of translational factors, the VPg precursors might facilitate the concentration of these factors next to the replication sites, thereby enhancing translation (12). Alternatively, the precursors might compete with VPg-linked RNA for the translational machinery providing a regulatory function during virus protein expression, replication, and packaging.

The final two proteins encoded in norovirus ORF1, 3CL Pro and polymerase, are the most genetically conserved and well-characterized calicivirus nonstructural proteins. The MNV-1 Pro and polymerase proteins share 71 to 73% amino acid sequence similarity with those of NV, SHV, CV, and MDV. Studies of vesiviruses showed that polymerase and Pro were produced in infected cells in the form of the ProPol precursor protein (29, 33, 45). The uncleaved 72-kDa ProPol protein was also detected in lagovirus (RHDV)-infected hepatocytes, along with comparable amounts of cleaved 58-kDa polymerase and 15-kDa Pro (21). Analysis of the bacterially expressed FCV ProPol demonstrated that the protein was an active proteinase and polymerase (22, 47).

Most of the data concerning expression of the norovirus proteinase and polymerase proteins have relied on *in vitro* studies of norovirus ORF1 polyprotein processing that have suggested an inefficient cleavage of ProPol into the mature proteinase and polymerase proteins. The cleavage was observed only under conditions of overexpression in bacteria (5, 26), during transient expression of the ORF1 sequence in cell culture (39), or when an ORF1 *in vitro* translational mixture containing active proteinase was incubated for an increased amount of time (4). Of interest, both forms of the norovirus polymerase (ProPol and Pol) were active when expressed in bacteria, suggesting possible differences in their functions and roles in virus replication (3, 10). In our study, detection of the 19- and 57-kDa proteins (corresponding to the mature Pro and Pol proteins, respectively) beginning early in infection (8 to 12 h p.i.) indicated efficient cleavage at the border of the MNV-1 Pro and Pol proteins. No more than a faint band corresponding to ProPol was observed among the immunoprecipitated proteins, suggesting that the ProPol form of Pro and Pol might play a transient role in MNV-1 replication. Additional pulse-chase labeling studies of MNV-1 protein synthesis are needed to further characterize the precursor-product relationship of the polymerase-related proteins. Elucidation of the functional differences of the two forms of virus polymerase should benefit from the development of a reverse genetics system for MNV.

Calicivirus proteinase and polymerase share not only sequence homology but also significant structural homology with the corresponding picornavirus proteins. The norovirus proteinase was found to have a folding similar to that of picornavirus chymotrypsin-like cysteine proteinases. The crystal structure of the enzyme (Chiba norovirus) determined at 2.8-Å resolution showed a protein composed of two N- and C-terminal domains with the active site located in the cleft between them (32). The active-site residues included conserved His30, Glu54, and Cys139, where His30 and Cys139 were recognized as catalytic residues and the function of Glu54 was described as being nonessential (32). Of interest, His30 and Cys139 are also conserved in the sequence of MNV-1 Pro; however, Glu54 is replaced with Asp54. The MNV-1 Pro sequence has a number of conserved amino acid residues found to be a part of the substrate binding site of the norovirus Pro protein (32); hence, the specificity of Pro for scissile bond recognition was found to be similar to that previously described for other noroviruses. The MNV-1 Pro protein recognized cleavage sites with glutamic acid and glutamine residues in the P1 position and alanine and glycine in the P1' position. In addition, the cleavage site established as the border of NTPase and p18 has a glutamic acid-asparagine dipeptide reminiscent of the FCV cleavage site between analogous proteins.

The elucidation of the norovirus processing strategy has relied on in vitro expression systems for the mapping of the Pro cleavage sites in the absence of a permissive cell culture system. This study is the first to compare the cleavage maps of the norovirus ORF1 polyprotein generated in an in vitro expression system and in infected cells. We found that the recombinant DNA-based expression systems, in general, properly identified the cleavage events that occur in cells. However, additional cleavage events occurred in cells, indicating that the physiologic processing of the MNV polyprotein involves both autocatalysis and host protein-mediated proteolysis.

In summary, five cleavage sites recognized by virus 3CL proteinase were identified in the murine norovirus ORF1 polyprotein. A comparison of these cleavage sites with those available for other caliciviruses showed similarities in proteolytic processing strategies that are conserved across the family *Caliciviridae*, and a unified system of nomenclature could prove useful. We propose that the murine norovirus proteins be numbered beginning at the N terminus of ORF1 as follows: NS1-2 (Nterm), NS3 (NTPase), NS4 (3A-like), NS5 (VPg), NS6 (Pro), and NS7 (Pol).

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