Replication of Murine Hepatitis Virus Is Regulated by Papain-Like Proteinase 1 Processing of Nonstructural Proteins 1, 2, and 3[▽]

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Coronaviruses are positive-strand RNA viruses that translate their genome RNA into polyproteins that are co- and posttranslationally processed into intermediate and mature replicase nonstructural proteins (nsps). In murine hepatitis virus (MHV), nsps 1, 2, and 3 are processed by two papain-like proteinase activities within nsp3 (PLP1 and PLP2) to yield nsp1, an nsp2-3 intermediate, and mature nsp2 and nsp3. To determine the role in replication of processing between nsp2 and nsp3 at cleavage site 2 (CS2) and PLP1 proteinase activity, mutations were engineered into the MHV genome at CS2, at CS1 and CS2, and at the PLP1 catalytic site, alone and in combination. Mutant viruses with abolished cleavage at CS2 were delayed in growth and RNA synthesis but grew to wild-type titers of $>10^7$ PFU/ml. Mutant viruses with deletion of both CS1 and CS2 exhibited both a delay in growth and a decrease in peak viral titer to $\sim 10^4$ PFU/ml. Inactivation of PLP1 catalytic residues resulted in a mutant virus that did not process at either CS1 or CS2 and was severely debilitated in growth, achieving only 10^2 PFU/ml. However, when both CS1 and CS2 were deleted in the presence of inactivated PLP1, the growth of the resulting mutant virus was partially compensated, comparable to that of the CS1 and CS2 deletion mutant. These results demonstrate that interactions of PLP1 with CS1 and CS2 are critical for protein processing and suggest that the interactions play specific roles in regulation of the functions of nsp1, 2, and 3 in viral RNA synthesis.

Murine hepatitis virus (MHV) is a member of the family *Coronaviridae* within the order *Nidovirales*. Coronaviruses have positive-strand RNA genomes ranging in size from 27 to 32 kb. The coronavirus genome RNA is also an mRNA, from which the first open reading frame, ORF1, is translated as a 500-kDa ORF1a polyprotein or, via a -1 ribosomal frameshift, as an 800-kDa ORF1ab fusion polyprotein (3, 7, 8, 29, 32). The polyproteins are co- and posttranslationally processed by virus-encoded proteinases into intermediate and mature proteins (Fig. 1A) that likely interact to mediate all viral RNA synthetic activities in the infected cell.

The number of proteinases encoded by ORF1 varies by coronavirus group. All coronaviruses encode a 3C-like proteinase (3CLpro) within nsp5 (30), responsible for processing nsps 4 through 16 (6, 16, 17, 31). The processing of nsps 1 through 3 is mediated by one or two papain-like proteinase (PLP) activities within nsp3 (4, 5, 19, 26, 45, 50). Group 1 and group 2a coronaviruses, such as human coronavirus 229E (HCoV-229E) and MHV, respectively, encode two PLPs, PLP1 and PLP2 (also known as PLpro1 and PLpro2, respectively). Group 2b and group 3 coronaviruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV) and infectious bronchitis virus (IBV), respectively, encode only one enzymatically active PLP in the position of PLP2. IBV possesses an inactive remnant of PLP1, while SARS-CoV does not possess a PLP1 sequence (42) (Fig. 1B). The observation that different coronaviruses successfully process nsps 1 to 3 using only one active

PLP has led some investigators to propose that PLP2 is a dominant or requisite cooperative proteinase activity even where PLP1 has been shown to be active in vitro (50).

In addition to the PLP domains, the 210-kDa nsp3 contains other conserved domains with demonstrated or predicted functions, including an “Ac domain” highly enriched in acidic residues; zinc ribbon motifs within the PLP domains (23, 51); an “X domain” with sequence homology to ADP ribose-1” monophosphate processing enzyme (Appr-1”-p), whose enzymatic activity has been shown in vitro for SARS-CoV and HCoV-229E (33, 34); and a C-terminal “Y domain” containing hydrophobic amino acid stretches that possibly confer membrane-spanning ability to this large protein. The structure of SARS-CoV core PLP domain of nsp3 has recently been solved, confirming many predicted features of the papain- and human TFIIS-based homology model proposed for the HCoV-229E PLP2 catalytic core (23). The substrate specificity of SARS-CoV PLP (P4-LXGG-P1) is shared by most coronavirus PLP2 enzymes and some PLP1 enzymes (44). The sequence specificity is restricted by large, hydrophobic, and/or aromatic residues surrounding the active site that are proposed to inhibit access of nonglycine amino acid side chains. PLPs without such strict requirements (such as MHV PLP1) do not possess these “gatekeeper” residues in the corresponding positions of their primary sequences.

Virus-mediated processing of the replicase polyprotein is required for the continued progression of viral RNA synthesis and, by extension, the production of progeny virions (1, 2, 28, 38, 39). Nidoviruses, including coronaviruses, employ a complex RNA synthesis program that functions to generate both new genomic RNA as well as a nested set of subgenomic mRNAs that are the translational templates for ORFs 2 to 7.

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FIG. 1. MHV genome organization and comparisons of coronavirus nsp1 to nsp4. (A) The MHV genome is 31.5 kb in length. The replicase gene comprises approximately 22 kb (ORF1a and ORF1b), while ORFs 2 through 7 (9.5 kb) encode structural and accessory proteins. The putative ORF1ab fusion polyprotein is shown, with mature nsp domains indicated by vertical lines and numbers. Viral PLP domains of nsp3 are shown as dashed boxes, and the viral 3C-like proteinase (nsp5) is indicated by a black box. Cleavage sites cleaved by PLPs and nsp5 are shown by corresponding arrowheads. CS1, CS2, and CS3 are indicated. (B) The organization of nsp1 to nsp4 is shown for the coronaviruses MHV, SARS-CoV, HCoV-229E, and IBV. PLPs are indicated by dashed boxes. Hatched gray box represents the inactive remnant of the IBV PLP1 domain. Other domains within nsp3 are indicated by white boxes: Ac, acidic domain; X, X domain (see text). Processing events confirmed *in vitro* or during infection are indicated by arrows.

Such a complex RNA synthesis program most probably requires multiple regulatory mechanisms, and the multidomain coronavirus nsp3 protein is a likely candidate for mediating this regulation, based on the predicted RNA synthesis and modification functions, as well as the cross-family and sometimes cross-order conservation of nsp3 domains. Studies using a replicon system for the arterivirus equine arteritis virus (EAV) have implicated the EAV nsp1, which is similar to the coronavirus nsp3, as a regulatory molecule. Studies involving mutagenesis of the metal-coordinating residues of the EAV zinc finger have revealed an essential role for this domain in the initiation of subgenomic RNA synthesis (46). While the zinc ribbon domains of the coronavirus nsp3 have not yet been implicated in RNA synthesis, *in vitro* studies with HCoV-229E have demonstrated that elimination of zinc ribbon metal coordination abolishes PLP1 activity (23).

For MHV, a link between polyprotein processing and viral RNA synthesis was first tested during virus infection by mutagenesis of cleavage site 1 (CS1) between nsp1 and nsp2 (18). The study demonstrated that, while cleavage between nsp1 and nsp2 was not required for viral replication, blocking this processing event resulted in a decrease in viral growth, RNA

synthesis, and cytopathic effect (CPE). In contrast to earlier studies of polyprotein processing that used inhibitors to block all proteinase activity (28), the study demonstrated that genetic impairment or elimination of specific polyprotein processing events could still yield infectious virus. CS1 is cleaved cotranslationally during infection with wild-type MHV, with no detectable nsp1-containing intermediate. In contrast, the second cleavage site, CS2, connects nsp2 (65 kDa) and nsp3 (210 kDa) in a 275-kDa intermediate protein (previously reported as p290 or p250) that is detectable in infected cells with a half-life of 30 to 60 min and ultimately processed at CS2 to yield nsp2 and nsp3 (14, 40). Both the intermediate nsp2-3 and the mature products nsp2 and nsp3 are simultaneously detectable, presumably due to the continuous translation of the replicase polyprotein throughout infection (14). Thus, it had not been determined whether proteolytic maturation of a confirmed intermediate nsp such as nsp2-3 could be blocked and still yield infectious virus.

In this study, we sought to determine whether proteolytic processing of the nsp2-3 intermediate protein at CS2 was required for MHV replication and whether processing at CS1 or CS2 could be mediated by PLP2 in the absence of PLP1 func-

TABLE 1. Primers used for mutagenesis of CS2 and PLP1

Primer name	Sequence ^a	Sense	Purpose
VURG1-15 Left	5'GGGTATACGGATTCTCGCTTACAGAATTCTGTT	+	Common primer, VURGs 1-15
VURG1 Right	5'AGGCCACCACAGGGAAACCTCCAG	—	Mutagenesis
VURG2 Right	5'AGGCCCGCGGAGGGAAACCTCCAG	—	Mutagenesis
VURG3 Right	5'AGGCCCGCTCCGGGAAACCTCCAG	—	Mutagenesis
VURG4 Right	5'AGGCCAGGACAGGGAAACCTCCAG	—	Mutagenesis
VURG5 Right	5'AGGCCAGTACAGGGAAACCTCCAG	—	Mutagenesis
VURG6 Right	5'AGGCCACGACAGGGAAACCTCCAG	—	Mutagenesis
VURG7 Right	5'AGGCCATGACAGGGAAACCTCCAG	—	Mutagenesis
VURG8 Right	5'AGGCCAGGTCCGGGAAACCTCCAG	—	Mutagenesis
VURG9 Right	5'AGGCCATGTCCGGGAAACCTCCAG	—	Mutagenesis
VURG10 Right	5'AGGCCACGTCCGGGAAACCTCCAG	—	Mutagenesis
VURG13 Right	5'AGGCAACGACAGGGAAACCTCCAG	—	Mutagenesis
VURG14 Right	5'AGGCTCGCACAGGGAAACCTCCAG	—	Mutagenesis
VURG15 Right	5'AGGCGCGCACAGGGAAACCTCCAG	—	Mutagenesis
VURG16 A	5'AGATCTATGGTTAAGCCATC	+	PCR Partner for VURG16 B
VURG16 B	5'CGTCTCGTCTT GGGAAACCTCCAGC	—	Mutagenesis for VURG16
VURG16 C	5'CGTCTCGAAGAAAGTCGAGTTTAAAC	+	Mutagenesis for VURG16
VURG16 D	5'ACTAGTTTGTCCACAAAGCATTGGTC	—	PCR partner for VURG16 C
VURG18/19 A	5'TTCGAAGGCGTGTTCAGAGTTTGAAAGTTGATAAA	+	PCR partner for VURG18/19 B
VURG18 B	5'GGTCTCGAACCATTAGTGCCTCTATAGCAGGC	—	Mutagenesis
VURG18 C	5'GGTCTCGGGTTGGCTCGTCTACTTTGATAGTA	+	Mutagenesis
VURG19 B	5'GGTCTCCCGGCATTAGTGCCTCTATAGCAGGC	—	Mutagenesis
VURG19 C	5'GGTCTCGGCCGCCCTGCGTTCTACTTTGATAGT	+	Mutagenesis
VURG18/19 D	5'GCGGCCGCCAGTGTGATGGATATCTGCAGA	—	PCR partner for VURG18/19 C

^a Underlining indicates nucleotides added for cloning purposes or nucleotides changed or deleted for mutagenesis.

tion. Mutant viral genomes were engineered encoding substitutions at CS2 that were predicted either to allow or to abolish cleavage, based on *in vitro* studies. Infectious mutant viruses were recovered with mutations at CS2, inactivation of PLP1, and deletions of both CS1 and CS2, as well as deletions of both cleavage sites and inactivation of PLP1. While mutant viruses with noncleaving mutations at CS2 showed delayed growth but wild-type peak titers, mutant viruses with abolished processing at both CS1 and CS2, either by cleavage site deletion or PLP1 inactivation, were both delayed and impaired in growth and RNA synthesis. These results indicate that regulated processing of nsps 1, 2, and 3 is essential for both the optimal timing and extent of viral RNA synthesis and productive virus infection.

MATERIALS AND METHODS

Wild-type virus, cells, and antibodies. MHV strain A59 or recombinant wild-type A59 (wtic) (18, 43) was used as the wild-type control in all MHV experiments. MHV-A59 reference sequences AY910861 and AY700211 were used for cloning studies. Delayed brain tumor (DBT) cells (24) and baby hamster kidney cells expressing the MHV receptor (BHK-MHVR) (11, 12) were grown in Dulbecco's modified Eagle medium that contained 10% fetal bovine serum for all experiments. Medium for BHK-MHVR cells was supplemented with G418 (0.8 mg/ml) for selection of cells expressing the receptor.

Most rabbit polyclonal antibodies used in biochemical experiments have been previously described. For MHV, these include α -nsp1 (VU221) (10), α -nsp2 (VU154) (41), α -nsp3 (VU164) (20), α -nsp8 (VU123) (6), and α -nsp13 (VUB1).

Construction of mutant MHV cDNA plasmids. Point mutations in the nsp2 and nsp3 coding regions were engineered using PCR and the primers shown in Table 1. For ABCD primer sets, primers A and B generated an A/B PCR product, and primers C and D generated a C/D PCR product. The MHV infectious cDNA fragment A construct (pCR-XL-TopoA), which consists of nucleotides (nt) 1 to 4882 was used as template DNA (49). Mutant fragment A constructs for MHV CS2 mutants (VURGs 1 to 16) were generated using a native BpmI site at the 3' end of the mutagenized segment at nt 3075 of pCR-XL-TopoA and a native BstZ17I site at the 5' end of the mutagenized segment at nt 1661 of pCR-XL-TopoA. Mutant segments were then cloned into

the pCR-XL-TopoA vector using these restriction sites. Mutant fragment A construct for the Δ CS1/ Δ CS2 mutant (VURG17) was cloned by excising the mutant CS2 site from the VURG16 fragment A construct using native 5' SacII and 3' BstZ17I sites and inserting it in place of the wild-type CS2 site in the CS1 deletion (Δ CS1) construct (18). Mutant fragment A constructs for the PLP inactivation mutants were constructed using the class II restriction enzyme method (49) in which PCR products A/B and C/D were ligated and were cloned into the vector at unique 5' BstBI and 3' EcoRV sites. Mutant fragment A construct for the Δ CS1/ Δ CS2 deletion/PLP1ko mutant (VURG21) was cloned by excising the mutant PLP1 site from the VURG19 construct and inserting it in place of the wild-type PLP1 site in fragment A using 5' BstBI and 3' EcoRV. Successful construct generation for all sites was confirmed by restriction digestion and sequencing.

Generation of MHV mutant viruses. Viruses containing PCR-generated mutations of the nsp2 and nsp3 coding sequences were produced using the infectious cDNA assembly strategies for MHV-A59 as previously described, with modifications (18, 43, 48, 49). Briefly, for MHV, plasmids containing the seven cDNA cassettes of the MHV genome were digested using MluI, BsmBI, and SfiI for fragment A, BglI and BsmBI for fragments B and C, BsmBI and NciI for fragments D and E, BsmBI for fragment F, and SfiI and BsmBI for fragment G. Digested, gel-purified fragments were ligated together in a total reaction volume of ~170 μ l overnight at 16°C. Following chloroform extraction and isopropanol precipitation of ligated DNA, capped, polyadenylated full-length RNA transcripts of MHV infectious cDNA were generated *in vitro* using the Message Machine T7 Transcription Kit (Ambion) following the manufacturer's protocol with modifications. Fifty-microliter reactions were supplemented with 7.5 μ l of 30 mM GTP, and transcription was performed at 40.5°C for 25 min, 37.5°C for 50 min, and 40.5°C for 25 min (B. Yount and R. S. Baric, personal communication). In parallel, capped, polyadenylated RNA transcripts encoding the MHV nucleocapsid protein (N) were generated *in vitro* using N cDNA generated from PCR (49). N transcripts and mutant MHV transcripts were then mixed and electroporated into BHK-MHVR cells. Briefly, BHK-MHVR cells were grown to subconfluence, trypsinized, and then washed twice with phosphate-buffered saline (PBS) and resuspended in PBS at a concentration of 10^7 cells/ml. Six hundred microliters of cells was then added to RNA transcripts in a 4-mm gap electroporation cuvette (BTX), and three electrical pulses of 850 V at 25 μ F were delivered with a Bio-Rad Gene Pulser Xcell electroporator. Transfected cells were then seeded onto a layer of 10^6 uninfected DBT cells in a 75-cm² flask and incubated at 37°C for 30 h. Virus viability was determined by CPE (syncytia formation), and progeny were passaged and clones were isolated by plaque assay. RNA was recovered from infected-cell monolayers using TRIzol (Invitrogen)

according to the manufacturer's instructions, and retention of introduced mutations was verified by reverse transcription-PCR and sequencing both prior to and following plaque purification.

Viral growth assays. For viral growth determination, DBT cells were infected with MHV wild-type, recombinant wild-type, and mutant viruses at the multiplicities of infection (MOIs) indicated for each experiment, from 1 to 5 PFU per ml (PFU/ml). For MHV, an MOI of 1 results in single cycle infection and was used where stocks of virus mutants were less than 10^7 PFU/ml. Following a 45-min adsorption with rocking at room temperature, medium was aspirated, and cells were washed three times with PBS and then incubated with prewarmed medium at 37°C. Aliquots of medium were collected from 1 to 24 h postinfection (p.i.), and virus titers were determined by plaque assay on DBT cells as described previously (28).

Protein immunoprecipitations. For protein labeling and immunoprecipitation experiments, cells were infected with MHV and incubated at 37°C. At 4.5 h p.i., medium was aspirated and replaced with medium lacking methionine and cysteine and supplemented with actinomycin D (Sigma) at a final concentration of 20 μ g/ml. At 6 h p.i., cells were labeled with [35 S]methionine-cysteine ([35 S]Met-Cys) at a concentration of 0.08 mCi/ml. Radiolabeled cells were lysed in 1 ml of lysis buffer without sodium dodecyl sulfate (SDS) (1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, and 50 mM Tris, pH 8.0) at 10 to 14 h p.i. Cellular debris and nuclei were pelleted by centrifugation at $1,500 \times g$ for 5 min at 4°C, and the supernatant was transferred to a fresh tube. One hundred microliters of cell lysate was subsequently used per 1 ml of immunoprecipitation reaction buffer. Lysates that were boiled prior to immunoprecipitation were boiled for 5 min in SDS at a final concentration of 1%. Lysate was combined with protein A-Sepharose beads and a 1:200 dilution of antibody in no-SDS lysis buffer supplemented with 1% SDS. After incubation at 4°C for 4 h, beads were pelleted and washed with low-salt lysis buffer (no-SDS lysis buffer with 150 mM NaCl) followed by high-salt lysis buffer (no-SDS lysis buffer with 1 M NaCl) and a final low-salt wash. After rinsing, 30 ml of 2 $\times$ SDS loading buffer (8% SDS, 0.2 M Tris, pH 8.8, 4 mM EDTA, 0.1% bromophenol blue, 40% glycerol, 0.5 M dithiothreitol) was added to the pelleted beads and boiled for 5 min prior to electrophoresis of the supernatant on 5 to 18% SDS-polyacrylamide gel electrophoresis (PAGE) gels or 4 to 12% Bis-Tris gels (NuPage, Invitrogen). In the case of electrophoresis on NuPage gels, proteins were eluted for 10 min at 70°C in 2 $\times$ LDS buffer (2 $\times$ LDS buffer contains 53 mM Tris-HCl, 70 mM Tris bare, 1% lithium dodecyl sulfate, 5% glycerol, 0.25 mM EDTA, 0.11 mM SERVA Blue G250, 0.0875 mM phenol red [pH 8.5])–1 $\times$ dithiothreitol loading buffer (Invitrogen) and run according to manufacturer's specifications.

For pulse-chase experiments, cells were infected as above. At 5.5 h p.i., cell supernatant was aspirated and replaced with medium lacking methionine and cysteine and supplemented with actinomycin D. At 6.5 h p.i., NaCl (final concentration, 200 mM) was added to the medium to prevent ribosomal initiation of translation and to effect synchronization of translated product. At 7 h p.i., medium was aspirated and replaced with medium containing actinomycin D and lacking Met-Cys, and cells were pulsed with [35 S]Met-Cys for 60 min. At 8 h p.i., one set of mock-infected or infected cell monolayers was harvested in radioimmunoprecipitation assay (RIPA) buffer (chase 0). The remaining set of mock-infected and infected cell monolayers was aspirated, washed, and supplemented with medium containing excess unlabeled methionine and cysteine. At 9.5 h p.i., this set of cells was harvested in RIPA buffer (chase 90). Images were processed using Adobe Photoshop CS2 (9.0). Images were analyzed using ImageJ (<http://rsb.info.nih.gov/ij/>).

Metabolic labeling of viral RNA. For metabolic labeling of MHV viral RNA, DBT cell monolayers ($\sim 1.5 \times 10^6$ cells) were either mock infected or infected at an MOI of 5 PFU/cell. Virus was adsorbed for 30 min at room temperature. Medium was then aspirated and replaced with fresh, prewarmed medium. Thirty minutes prior to labeling, actinomycin D was added to the cells at a final concentration of 20 μ g/ml. Cells were labeled with 30 μ Ci of [3 H]uridine in the presence of actinomycin D for the time intervals indicated for each experiment. Cells were washed once with PBS and then lysed with 700 μ l of no-SDS lysis buffer. Lysates were centrifuged at $1,500 \times g$ to remove nuclei, and then RNA in 200 μ l of cytosolic extract was precipitated using trichloroacetic acid. Precipitated RNA was dried onto glass microfiber filters (Whatman) using vacuum filtration, and radioactivity was measured in a liquid scintillation counter (Beckman). Quantitative analysis was performed using ImageJ (<http://rsb.info.nih.gov/ij/>).

RESULTS

Changes at CS2 allow recovery of infectious viruses. The determinants for processing at the nsp2 $\downarrow$ nsp3 cleavage site, CS2, had been defined in vitro but had not been tested during

FIG. 2. Construction of MHV CS2, CS1/CS2, and PLP1 mutant viruses. (A) nsps1 to 3 are shown, with cleavage sites 1 and 2 indicated. The P3 through P3' residues of CS2 are expanded below. VURG mutants are indicated by number and by amino acid substitution beneath the corresponding P2, P1, and P1' residues. Deletion of P2-P1' (CysAlaGly) of CS2 in VURG16 is indicated by the caret. (B) The nucleotide and amino acid (aa) locations of PLP1 catalytic Cys1121 and flanking Trp1122 residues are indicated. VURG mutants are shown below with deletion of CS1 (Δ CS1) and CS2 (Δ CS2), substitution of Cys1121, or substitution of both Cys1121 and Trp1122.

MHV infection of cells in culture (5). To test requirements for processing nsp2-3 into nsp2 and nsp3, mutations were introduced in the genome that resulted in substitutions at the P2, P1, and P1' sites of CS2 (Fig. 2). Following electroporation of in vitro transcribed mutant genomic RNA into BHK-MHVR cells, all cultures exhibited typical viral CPE of syncytia within 24 h, and all cultures were completely involved in syncytia by 48 h postelectroporation. When stocks of viruses were se-

FIG. 3. Processing of CS2 mutants. (A) Pulse-label. DBT cells were mock infected or infected with the indicated viruses as described in Materials and Methods. Proteins were radiolabeled for 1 h, from 7 to 8 h p.i. Cell lysates were immunoprecipitated with antibodies against nsp1, nsp2, or nsp3, resolved by 5 to 18% SDS-PAGE, and imaged by fluorography. Mock, mock-infected cells; A59, MHV-A59 lab stock. VURG mutants are indicated by number, with an asterisk indicating in vitro cleavage of CS2 mutations. Locations of nsp1, nsp2, nsp3, and nsp2-3 are shown to the right of the gel. (B) Pulse-chase. For each virus, duplicate DBT cell monolayers were mock infected or infected with the indicated viruses and were radiolabeled with [35 S]methionine-cysteine from 7 to 8 h p.i. Following a 60-min pulse, one set of plates was harvested in RIPA buffer (0). The second set of plates was washed and supplemented with complete medium lacking radiolabel and incubated an additional 90 min. Viral proteins were immunoprecipitated using α -nsp2 antibodies, and proteins were resolved by SDS-PAGE. Molecular weight markers and antibodies are indicated to the left of each gel panel.

quenced across the cleavage site and the 400 nt flanking each side of the cleavage site, all viruses had retained the engineered mutations, both prior to and following plaque purification. While some CS2 mutants exhibited plaque sizes substantially smaller than those of wild-type virus (VURGs 4, 8, 9, and 16), plaque sizes were homogeneous for each mutant both prior to and following plaque purification (data not shown).

CS2 mutants differ in their processing phenotypes. Our previous studies of the nsp1 $\downarrow$ nsp2 cleavage site (CS1) demonstrated a direct correlation between small plaque size and inhibition of cleavage (18). Ten of 14 mutants with changes at CS2 had plaque sizes indistinguishable from those of wild-type virus, suggesting a possible correlation between plaque size and CS2 processing. To assess this, we examined processing phenotypes for these viruses. DBT cells were either mock infected or infected with wtic or CS2 mutant viruses (VURGs 1 to 16), and cells were then labeled with [35 S]Met-Cys. Cytoplasmic extracts were prepared and immunoprecipitated with antibodies against nsp1, nsp2, and nsp3 (Fig. 3A). Based on in vitro results, VURG1 (P1-Gly) and VURG2 (P2-Ser) mutants were predicted to allow cleavage. During infection, both mutants processed nsp2 and nsp3 with the same timing and com-

pleteness as wild-type virus. In contrast, the other CS2 mutants had processing at CS2 that varied from 0% to 100% compared to wild-type virus. The VURG4, -8, -9, and -16 mutants produced a nonprocessed nsp2-3 protein and little or no detectable mature nsp2 or nsp3. To test whether CS2 processing in these mutants was delayed rather than abolished, pulse-chase analysis of nsp2-3 expression and processing was performed (Fig. 3B). wtic and VURG1 (P1-Gly) had the same pattern of nsp2 expression, showing small amounts of nsp2 following the 60-min pulse (chase 0) and greater amounts following the 90-min chase (chase 90). In all noncleaving mutants, nsp2-3 was not further processed during the chase period.

Other substitutions only partially blocked cleavage at CS2 during infection of cells (VURGs 3, 7, 10, 13, 14, and 15). In general, substitutions in the virus that recapitulated the substitutions tested in vitro demonstrated similar processing phenotypes. In particular, VURG3 (P2-Gly) closely recapitulated the in vitro phenotype, processing 22% of CS2 compared to wild-type virus. A notable exception to the similarities between the effect of substitution in vitro and in the context of infection was the P1-Ala residue of CS2. While in vitro studies indicated that the P1-Ala residue of CS2 required a small, uncharged

FIG. 4. Growth of CS2 mutants on DBT cells. DBT cells were infected at an MOI of 5 PFU/cell with the indicated viruses as described in Materials and Methods: VURG1 (P1-Gly), VURG4 (P1-Pro), VURG8 (P2P1-GlyPro), and VURG16 (Δ CS2). At the times indicated postinfection, aliquots of supernatant were obtained, and viral titers were determined by plaque assay on DBT cells. Peak titers (PFU/ml) were 6.7×10^7 (wtic), 7.3×10^7 (VURG1), 5.6×10^7 (VURG4), 5.6×10^7 (VURG8), and 6.7×10^7 (VURG16). Data points are the means of two samples each of two concurrent independent replicate experiments for each virus.

amino acid to maintain cleavage (5), substitution in the virus at P1 by Thr, Arg, or His maintained CS2 cleavage at or near wild-type levels. Additionally, there were clearly nontolerated changes. Specifically, P2, P1, and P1' residues could not be deleted, double substitutions of P2-P1 could not be cleaved efficiently, and substitution of proline at the P1 position abolished cleavage. Otherwise, even bulky or charged residues at P1 or P1' allowed at least partial cleavage at CS2. These results suggest that there is significant flexibility at these residues for recognition and processing by viral proteinases during polyprotein expression and processing in viral infection.

CS2 noncleaving mutants are delayed, but not decreased, in viral growth. To determine the effect of alterations at CS2 on viral growth, single-cycle growth assays were performed using wtic and the cleaving mutant, VURG1 (P1-Gly), in direct comparison to mutant viruses with near-complete or complete blocks of CS2 processing (VURGs 4, 8, and 16) (Fig. 4). DBT cell monolayers were infected with the indicated viruses at an MOI of 5 PFU/cell, and supernatant was saved and assessed for titer by plaque assay at specific times postinfection. The VURG1 mutant, which allowed cleavage at CS2, grew with identical kinetics and peak titer as wtic. In contrast, noncleaving mutant viruses exhibited a 4-h delay in the detection of measurable infectious virus but ultimately achieved peak titers comparable to wtic, though achievement of peak titer was also delayed by 4 h. Thus, the defect in protein processing displayed by mutants with abolished cleavage at CS2 was associated with

FIG. 5. RNA synthesis of CS2 mutants. DBT cells were infected with the indicated viruses at an MOI of 5 PFU/cell as described in Materials and Methods. At 30 min prior to addition of metabolic label, medium was aspirated and replaced with prewarmed complete medium containing 20 μ g/ml actinomycin D. At the indicated times postinfection, 0.03 mCi/ml of [3 H]uridine was added to the medium for 2 h, and at the end of the labeling period, cells were harvested in RIPA buffer. Total actinomycin D-resistant RNA was precipitated in 10% trichloroacetic acid and quantitated by liquid scintillation counting. Data points are the means of two samples each of two concurrent independent replicate experiments for each virus.

a delay in production or release of progeny virions into the supernatant.

CS2 noncleaving mutants are delayed in viral RNA synthesis. To determine if the delay in virus growth in CS2 noncleaving mutants was associated with impaired viral RNA synthesis, a time course assay of viral RNA synthesis was performed. DBT cells were mock infected or infected with wtic or cleaving mutant VURG1 or with noncleaving mutants 4, 8, or 16. Newly produced viral RNA was metabolically labeled with [3 H]uridine in the presence of actinomycin D for nonoverlapping 2-h intervals between 3 and 15 h p.i., and RNA was quantitated by scintillation counting (Fig. 5). The pattern and extent of RNA synthesis were the same for wtic and VURG1, peaking at 9 to 11 h p.i. Noncleaving mutants VURG4, VURG8, and VURG16, however, were delayed in the synthesis of total RNA, producing the greatest amounts of RNA from 11 to 13 h p.i. The delay in peak RNA synthesis was consistent with the delay in detection of infectious virus particles during growth, suggesting that the delay in growth was due to changes in the initiation or amplification of RNA synthesis rather than specific defects in virus assembly or release.

Deletion of CS1 and CS2 allows the recovery of infectious virus that does not process either cleavage site. In light of results presented above and in our earlier study involving the CS1 cleavage site (18), we hypothesized that processing at both CS1 and CS2 could be abolished and still allow the production of infectious virus. To test this hypothesis, a construct was engineered in which both CS1 and CS2 were deleted (VURG17) (Fig. 2B). Following electroporation of full-length genomic RNA containing both mutations, cells exhibited pro-

FIG. 6. Polypeptide processing of cleavage site and proteinase combination mutants. DBT cells were mock infected or infected with the indicated viruses. At 4 h p.i., cells were labeled with [35 S]Met-Cys until 12 h p.i., when cell lysates were harvested, and proteins were immunoprecipitated with antibodies against nsp1, nsp2, nsp3, and nsp8. Proteins were then resolved by SDS-PAGE. Mock, mock-infected cells. The nsps detected are indicated to the right of the gels. Lane numbers 1 to 7 below the gel are referenced in the text. Molecular weight markers are indicated to the left of the gels. Antibodies used are indicated to the left of each panel. Lanes 5, 6, and 7 (VURGs 17, 19, and 21) are in separate boxes for anti-nsp3 and anti-nsp8, since these were exposed three times longer than lanes 1 to 4 to account for decreased protein expression.

ductive CPE by 24 h postelectroporation. Involvement of the entire monolayer in syncytia was slower than that observed for CS2 mutant viruses, and subsequent infections with supernatant isolated from the original electroporated culture corroborated that observation. Sequencing of RNA isolated from infections with this virus confirmed the deletion of both cleavage sites.

To determine if VURG17 was capable of processing nsp1, nsp2, and nsp3, DBT cells were infected, and radiolabeled viral proteins were detected by immunoprecipitation of cell lysates using antibodies against nsp1, nsp2, and nsp3, as well as nsp8 as a control for 3CLpro-mediated processing (Fig. 6, lanes 1 to 5). The results were compared to wild-type virus (lane 2), the CS1 deletion mutant (Δ CS1, lane 3) and the CS2 deletion mutant (VURG16, lane 4). Processing by 3CLpro was intact for all wild-type and mutant viruses, as indicated by the presence of mature nsp8 3CLpro cleavage product. During wtic infection, mature nsp1 (28 kDa), nsp2 (65 kDa), and nsp3 (210 kDa) were detected, as was the intermediate nsp2-3 (275 kDa). For the Δ CS1 mutant, while nsp1, nsp2, and nsp2-3 were not detected, nsp3 was detected, as well as a new nsp1-2-3 precursor (303 kDa) and a noncleaved nsp1-2 protein (93 kDa; this protein is detected either as a single band or as a doublet with no apparent correlation with differing experimental conditions). For VURG17 (lane 5), no mature nsp1, nsp2, nsp3, or intermediate nsp2-3 proteins were detected; rather, only a 303-kDa nsp1-2-3 precursor was detected. Compared with the Δ CS1 and VURG16 alone, the amount of protein produced

during VURG17 infection was reduced, consistent with the decreased growth. These results clearly demonstrate that processing of neither CS1 nor CS2 is essential for viral replication, and that a noncleaved nsp1-2-3 fusion protein can mediate all necessary roles in the viral life cycle.

Catalytic inactivation of PLP1 allows recovery of virus with severe impairment of growth. Based on the observation that processing at both CS1 and CS2 can be eliminated and still yield infectious virus, we hypothesized that PLP1 proteinase activity is not essential for MHV replication. Since deletion of the entire PLP1 domain of nsp3 might have unintended effects on nsp3 conformation or RNA structure, we chose to engineer substitutions at catalytic Cys1121, an approach which has been shown to inactivate PLP1 proteinase function *in vitro* (51). In VURG18, a single-nucleotide mutation (nt 3570) resulted in a Cys1121Gly substitution. In VURG19, nt 3570 to 3575 were mutated, resulting in combined Cys1121Ala and Trp1122Ala substitutions (Fig. 2B). In VURG20, nt 3570 to 3575 were deleted, resulting in the deletion of the Cys1121 and Trp1122 and juxtaposition of Asn1120 and Leu1123 (not shown).

Following electroporation of the full-length mutated genome RNA, a low-level, nonprogressive CPE (<20 cells/syncytia) was observed beginning at ~18 h postelectroporation. The low-level nonprogressive CPE continued for 7 days, during which no infectious virus could be detected in supernatant medium of transfected monolayers. At day 7 postelectroporation, cells in both VURG18 and VURG19 electroporations exhibited spreading and productive CPE, and fresh DBT cells inoculated with supernatant from these flasks likewise exhibited CPE within 24 h. Cells that were electroporated with the mutant genome containing the deletion of the Cys1121/Trp1122 dipeptide (VURG20) did not exhibit signs of productive CPE, and the limited nonprogressive syncytia formation that was observed on days 1 and 2 postelectroporation had disappeared by day 10, when the cells were discarded. Sequencing of passage 1 virus from VURG18 and VURG19 verified the retention of the intended mutations at the PLP1 catalytic site. No other mutations in the 400 nt flanking either side of the catalytic site were detected.

When VURG18 and VURG19 were passaged in DBT cells, both viruses initially grew very poorly in culture, with plaques detectable only in either nondiluted or 1:10 diluted medium, corresponding to titers of $\leq 10^2$ PFU/ml (data not shown). Following two rounds of passage of supernatant on fresh DBT cells, VURG18 (Cys1121Gly) exhibited a recovery of titer to $>10^7$ PFU/ml, and sequencing across the initial site of mutation demonstrated that VURG18 had reverted to wild-type Cys1121 by reversion of the single nucleotide mutation. In contrast, VURG19 maintained its 6-nt mutation and did not recover a more efficient growth phenotype following two rounds of passage. Therefore, VURG19 was used in subsequent experiments of growth and protein processing.

To determine if VURG19 was capable of processing at CS1 and CS2, DBT cells were infected, and cell lysates were immunoprecipitated as for VURG17 infection (Fig. 6, lane 6). Infection with VURG19 resulted in a pattern of protein expression and processing indistinguishable from that of VURG17 (lane 5), with diminished protein expression, no detectable nsp1, nsp2, nsp3, or nsp2-3, and a new nsp1-2-3 precursor. Since no higher-molecular weight products were

FIG. 7. Growth of cleavage site and proteinase combination mutants. DBT cells were infected at an MOI of 1 PFU/cell with the indicated viruses as described in Materials and Methods. At the times indicated postinfection, aliquots of medium were obtained, and titers were determined by plaque assay on DBT cells. Data points are the means of two samples each of two concurrent independent replicate experiments for each virus. Growth of VURG19 was at or below the level of detection, but an estimate of growth is illustrated by a dotted line for comparison.

detected with nsp3 antiserum, processing by PLP2 between nsp3 and nsp4 at CS3 was presumably intact, as was processing by 3CLpro at nsp8. The results demonstrate that PLP1 proteinase activity is not absolutely required for MHV replication in culture. These results also constitute the first genetic confirmation of the activity of PLP1 at these sites during virus infection. In addition, these results demonstrate that in the presence of a proteolytically inactive PLP1, the MHV PLP2 is not able to cleave at either CS1 or CS2 but retains its activity at CS3.

Inactivation of PLP1 in the presence of the deletion of CS1 and CS2 yields virus that does not process nsp1, nsp2, or nsp3 but is compensated in growth. It was clear that although VURG17 and VURG19 mutants had the same protein processing phenotype, VURG19 was much more delayed in emergence following electroporation and subsequently in growth in culture. A possible explanation for this difference was that the VURG19 still allowed binding of the inactive proteinase to CS1 and/or CS2 but not processing and release of the cleaved proteins, resulting in even more impaired protein function. If so, then the folding of the noncleaved nsp1-2-3 precursor might impair other functions of these proteins. This would not be the case for VURG17, since the deletion of the cleavage site would limit or eliminate binding of the active PLP1 to the altered sites. To test this possibility, a mutant construct was engineered which encoded both VURG17 and VURG19 mutations (VURG21) (Fig. 2B). Following electroporation of

VURG21 full-length genomic RNAs, cells exhibited productive CPE within 24 h, similar to VURG17. Involvement of the entire monolayer in viral syncytia was delayed compared to VURG16, but it was much more rapid and extensive than VURG19.

When the protein processing phenotype of VURG21 was compared to VURG17 and VURG19 (Fig. 6, lane 7), it was found to be identical to both of them, with no detectable nsp1, nsp2, nsp3, or nsp 2-3 but with a detectable nsp1-2-3 precursor, as well as evidence of PLP2 cleavage at CS3 and intact 3CLpro cleavage at nsp8. VURG21 was then directly compared with wtic, Δ CS1, VURG16, and VURG17 viruses in a growth assay (Fig. 7). VURG19 was not directly assessed in this experiment because of its extreme growth impairment, with consistently delayed and low-level growth of less than 10^2 PFU/ml, even after extended infection of >30 h. In Fig. 7, an estimate of its growth phenotype is included merely for comparison. As observed in previous studies (18), infection with the Δ CS1 mutant resulted in a 1-log₁₀ reduction in peak titer but no delay. In contrast, infection with VURG16 resulted in the delay of viral burst but an eventual achievement of near-wild-type growth. The growth of VURG17 exhibited both a delay in growth as well as a 1-log₁₀ reduction in peak titer. The growth phenotype of VURG21 was the same as that of VURG17. Taken together, these results suggest that, while catalytic inactivation of PLP1 results in extreme impairment of viral growth, deletion of CS1 and CS2 along with the inactivation of the PLP removes a substantial block in viral replication, restoring viral growth to $>10^5$ PFU/ml.

DISCUSSION

In this report, we have shown that MHV has substantial flexibility in cleavage site and proteinase interactions around nsp1, nsp2, and nsp3 that can still allow virus replication in culture. While our previous work showed that CS1 and even the entire nsp2 could be deleted in viable mutant viruses, the present study demonstrates that all cleavages mediated by PLP1 can be eliminated, as can the proteinase activity of PLP1. However, this study also shows that while a noncleaved nsp1-2-3 precursor can mediate all required steps in virus replication, the regulation of polyprotein processing into intermediate and mature forms of nsp1, nsp2, and nsp3 is important for both the timing and extent of viral RNA synthesis and the production of infectious virus particles.

The processing network of PLP1, CS1, and CS2. The coronaviruses use either one or two PLP activities in nsp3 to process the first three cleavage sites in the replicase polyprotein, CS1, CS2, and CS3, in order to liberate intermediate and mature forms of nsp1, nsp2, and nsp3. In vitro studies have demonstrated that there are combinations of independent, dominant, and potentially cooperative activities of PLP1 and PLP2 at CS1, CS2, and CS3 for the human coronavirus HCoV-229E (51). In vitro results indicate that the 229E PLP2 is able to cleave at CS2 in the absence of PLP1 function, while an inactive PLP2 inhibits the activity of PLP1 at CS2. Thus, it was proposed that PLP2 is the dominant proteinase responsible for cleavage at CS2 and CS3, while PLP1 likely serves as the dominant or only proteinase for cleavage at CS1 (51). We tested this model during MHV infection by comparing the

processing of CS1, CS2, and CS3 in wild-type virus and mutants with deletion or inactivation of CS1, CS2, and PLP1. VURG17 and VURG19 had the same processing phenotype, with no cleavage at CS1 or CS2 but with intact processing at CS3. VURG19 demonstrated that processing at CS3 can occur in the absence of active PLP1, supporting *in vitro* results (27) and providing the first genetic confirmation in a virus that PLP2 alone is responsible for cleaving CS3.

In contrast, there was no evidence of cleavage at either CS1 or CS2 in VURG19 infection. This result argues strongly that during MHV infection in culture, PLP1 is the dominant or only proteinase responsible for cleavage at CS1 and CS2 and that there is no cooperative processing by PLP2 at either CS1 or CS2. This result is different from that observed *in vitro* with HCoV-229E PLP1 and PLP2 cleavage of substrates containing CS1 and CS2 (51). There are several possible explanations for the differences. The nsp1, nsp2, and nsp3 of MHV and HCoV-229E are from different coronavirus groups, have different sizes, and have limited amino acid similarity. In addition, the HCoV-229E CS1 and CS2 conform more closely to the proposed recognition sites of PLP2 than do the CS1 and CS2 of MHV. Thus, it is possible that MHV PLP1 and PLP2 interactions with CS1 and CS2 differ from those of 229E. However, the observations obtained with 229E constructs were not confirmed in the context of infection as were our results with MHV, and, thus, the differences may reflect the comparative complexity of the environment in which the protein expression and processing occurs. Thus, it is possible that *in vitro* results for any coronavirus may demonstrate potential activities and interactions that do not occur during virus infection.

The experiments in this study do not rule out the possibility that PLP2 is cooperating with PLP1 in cleavage of CS1 or CS2 or that PLP2 has the innate capacity to cleave these sites alone. It is possible that the Cys1121Ala and Trp1122Ala substitutions of VURG19 could alter an existing cooperative interaction of PLP2 with PLP1 at CS1 or CS2. Alternatively, it is possible that inactive PLP1 could retain the ability to interact with or bind to CS1 or CS2 without effecting cleavage and thereby mask or block any cooperative or compensatory activity of PLP2 at these sites. Testing this hypothesis would require deletion of the PLP1 domain in the presence of intact CS1, CS2, and PLP2. This might prove difficult, based on the fact that we were unable to recover a mutant with deletion of only Cys1121 and Trp1122, arguing that other functions or critical structural roles may be mediated by the PLP1 domain of nsp3. Regardless, it remains clear that during infection, PLP2 does not have dominant or compensatory cleavage activity in the presence of inactive PLP1.

Determinants of processing at CS2. Determinants of processing at CS2 have been examined *in vitro* (5), and the studies revealed somewhat less stringent requirements than those defined at CS1 (19, 25), though results did suggest a requirement for a small, uncharged residue at the P1 position (5). In our current study, substitution or deletion at CS2 P2 and P1' likewise showed tremendous flexibility for substitutions that still allowed cleavage, in several cases matching the efficiency of the wild-type CS2 sequence. While many substitutions recapitulated the partial processing phenotypes observed during *in vitro* studies, a notable exception was the P1 position. In contrast to the apparent requirement for a small amino acid at P1

for CS2 cleavage *in vitro*, in the context of infection, nonconservative changes were tolerated at P1, and mutant viruses still retained cleavage efficiency at or near wild-type levels, including replacement of P1-Ala with Arg, Thr, or His. In the face of this flexibility at CS2, there were some limitations on changes, specifically that the P2, P1, and P1' residues could not be deleted.

The reasons for the differences in CS1 and CS2 tolerance for substitutions is not obvious, nor is it apparent why there are different requirements at the P1 residue of CS2 *in vitro* and during infection. The answer may lie in differing requirements in the larger context of the flanking P5-P5' residues of each cleavage site, differing requirements for processing from the nascent polypeptide in the context of infection, and possibly in other requirements for protein functions or interactions *in vivo* during infection of animals. Finally, it is possible that the differences are a manifestation of a requirement that CS1 is cleaved cotranslationally, followed by the ordered, posttranslational processing of a nsp2-3 precursor into mature nsp2 and nsp3.

Protein processing and the regulation of viral replication.

The results in this report support the conclusion that there is an intimate link between PLP1 activity, processing of the nsp1-2-3 precursor, and regulation of viral RNA synthesis. There is precedent for such regulation in other positive-strand RNA viruses, specifically the alphaviruses, in which negative-strand and positive-strand RNA synthesis is regulated by the processing of the replicase polypeptide P1234 (13, 22, 37, 47). In the coronaviruses, the ORF1a and ORF1ab polypeptides are processed into several intermediate proteins prior to maturation cleavage of nsp1 to nsp16 (15, 18, 20, 21, 40). Most relevant to this report, MHV nsp1 is cotranslationally cleaved at CS1, followed by PLP2-mediated cleavage at CS3 between nsp3 and nsp4. Noncleaved nsp2-3 is detected as an intermediate with a half-life of 30 to 60 min during infection but ultimately is processed by PLP1 at CS2 to liberate nsp2 and nsp3 proteins. Interestingly, our previous study showed that mutant viruses in which nsp2 had been deleted and which contained a chimeric CS1-CS2 cleavage site between nsp1 and nsp3 had decreased overall RNA synthesis and growth but no delay in RNA synthesis or extension of the eclipse phase of virus growth (20). In that study, processing of the chimeric site between nsp1 and nsp3 appeared to be cotranslational, with no intermediate nsp1-3 precursor detected. Together, these studies suggest that processing at CS1 is critical for the amount of RNA synthesis and new infectious virus, while processing at CS2 is involved in regulation of the timing and progression of RNA synthesis and production of infectious virus. The observation that nsp2 can be completely deleted further suggests that release of mature nsp3 may be the critical event regulated by processing of CS2.

Integration of proteolytic processing and RNA synthesis during MHV infection. VURG17 and VURG19 had identical protein processing phenotypes, but VURG19 was profoundly growth impaired in comparison to VURG17, a growth impairment that was compensated in the combination mutant, VURG21. Since there are several confirmed and predicted functions in nsp3 in addition to proteinase activities (33–36) and since all of nsp2 and the carboxy-terminal half of nsp1 can be deleted in viable mutants (9, 20), we propose that the observed differences in the mutant viruses are due to changes

in nsp3 functions resulting from differential folding of nsp1-2-3. According to this model, in VURG17, the active PLP1 would not recognize or interact with the polyprotein at the deleted CS1 or CS2 sites, and the noncleaved nsp1-2-3 protein could affect the delayed and impaired, but ultimately complete, RNA synthesis program required for productive infection. This model further predicts that VURG19, while abolished in PLP1 catalytic activity, would retain the capacity to recognize and interact with intact CS1 and/or CS2, further impairing the residual function of the nsp1-2-3. Finally, this model would account for observation that VURG21 has the same growth phenotype as VURG17, since the deletion of CS1 and CS2 would presumably eliminate recognition by the inactive PLP1 and restore the sufficient, albeit impaired, functions of nsp1-2-3.

In contrast to the detection of viral CPE within 1 to 2 days for VURG17 and other CS2 mutants, the first detection of VURG19 productive viral CPE and measurable virus required 7 days after electroporation of the mutant genome RNA. However, once CPE and virus were detectable, VURG19 reproducibly formed plaques and grew consistently at $\sim 10^2$ PFU/ml. This late detection indicated that extremely low levels of replication were occurring and that the emergence of VURG19 required an adaptive response under selective pressure. The rapid reversion of the single-nucleotide mutation in VURG18 supports this likelihood. Since inactivation of CS1 and CS2 in VURG21 partially compensated the defect of VURG19, we sequenced VURG19 across the cleavage site coding region; however, no coding or noncoding changes were identified (data not shown). Thus, we propose that other compensating mutation(s) have been selected in VURG19 that are distinct from PLP1, CS1, or CS2. We are currently determining if the further compensatory adaptation can be selected during passage and if specific changes are present across the entire virus genome. Overall, the ability to recover and analyze mutant viruses with stable changes in cleavage sites and proteinases provides a powerful tool to define interactions and functions of nsp1, nsp2 and nsp3, their precursors, and the functional group that includes the proteinases, cleavage sites, and regulated processing of these proteins.

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